IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicant:	Ebrahim ZANDI, *et al.*
Title:	COMPOSITION AND METHOD FOR RECONSTITUTING IkB KINASE IN YEAST AND METHODS OF USING SAME
Appl. No.:	10/079,949
Filing Date:	2/19/2002
Office:	Prouty, Rebecca E.
Art Unit:	1652
Confirmation Number:	6542

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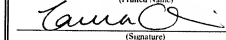
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April 5, 2010
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BRIEF ON APPEAL

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Sir:

This Appeal Brief is further to the Notice of Appeal, filed February 2, 2010, for the above-noted application. A Notice of Panel Decision from Pre-Appeal Brief Review was mailed March 3, 2010, thereby making the Brief on Appeal due April 3, 2010. However, as April 3, 2010 is a Saturday, this Brief is timely filed by the next business day, namely April 5, 2010.

A Final Office Action was mailed by the U.S. Patent and Trademark Office for the above-noted application on September 2, 2009 and a Notice of Appeal from this Action was timely filed on February 2, 2010 in light of a Petition for a Two Months Extension of

Time with payment of the fee. The rejection of the present application is ripe for appeal by virtue of the claims having been previously rejected twice, thus satisfying the regulatory requirement that the claims be twice rejected.

Under the provisions of 37 C.F.R. § 41.37, this Appeal Brief is being filed together with a credit card payment form in the amount of $270.00 covering the 37 C.F.R. § 41.20(b)(2) appeal fee for a small entity. If this fee is deemed to be insufficient, authorization is hereby given to charge any deficiency (or credit any balance) to the undersigned deposit account 19-0741.

REAL PARTY IN INTEREST

The real party in interest is the University of Southern California. An assignment of rights from the inventors and Appellants, Ebrahim Zandi and Beth Schomer Miller, to the University of Southern California was recorded at the United States Patent and Trademark Office on May 31, 2002, at Reel 012943 and Frame 0139.

RELATED APPEALS AND INTERFERENCES

None.

STATUS OF CLAIMS

Canceled claims: 1, 3, 4, 8-16, 20 and 24-41.

Pending claims: 2, 5-7, 17-19, 21-23 and 42.

Withdrawn claims: No claims are withdrawn.

Rejected claims: 2, 5-7, 17-19, 21-23 and 42.

Appealed claims: 2, 5-7, 17-19, 21-23 and 42.

Allowed claims: No claims are allowed.

Objected claims: No claims are objected to.

STATUS OF AMENDMENTS

Appellants filed an amendment and a Request for Continued Examination on July 28, 2009 in response to the Final Office Action dated June 9. 2009. In reply, a Final Office Action was issued on September 2, 2009. Appellants filed a supplemental amendment on December 1, 2009, which was refused entry by the Office in an Advisory Action issued on December 29, 2009.

The appealed claims and their status identifiers reflect those submitted in the Amendment and Reply filed July 28, 2009, and which were pending before the Examiner when the Final Office Action was issued on September 2, 2009.

SUMMARY OF CLAIMED SUBJECT MATTER

The claimed subject matter, in general, relates to preparing an activated IKK protein complex in yeast by transforming yeast with sequences encoding the three subunits of the IKK protein complex - IKKα, IKKβ, and IKKγ. As of the effective filing date of the present application, it was believed that activation of the IKK complex requires the TNF-α and NF-κB signaling pathways (page 3, line 12 to page 4, line 12), which are present in mammalian cells but absent in yeast (page 6, lines 6 to 7). The TNF-α and NF-κB signaling pathways are highly complicated and not fully understood mammalian pathways and thus an effort to reconstitute them in yeast would likely be unsuccessful. Accordingly, one of skill in the art at the time the invention was made would not have expected that an activated IKK complex could be prepared in yeast, which lacks the TNF-α and NF-κB signaling pathways.

The present invention, however, provides a ready solution to this problem by demonstrating that the subunit IKKγ regulates autophosphorylation of the subunit IKKβ leading to self-activation of the IKK complex. *See*, Experimental Example 2 (page 15, line 29 to page 17, line 19, in particular page 16, lines 20 to 21) and Summary of Invention (page 7, lines 6 to 14). Therefore, an activated IKK complex can be prepared in yeast by simply expressing the subunit proteins which would then undergo autophosphorylation and self-activation, without having to reconstitute the TNF-α and NF-κB signaling pathways in the yeast. This surprising discovery then is the basis of the presently claimed method of preparing substantially homogenous, biologically functional and activated IKK protein complex in yeast.

Claims 2, 5-7, 17-19, 21-23 and 42 remain pending and are subject to appeal. The present application presents two independent claims, namely, claims 2 and 42. Claims 5-7, 17-19 and 21-23 depend from claim 2 or claim 42.

Independent claim 2 is directed to a method for preparing substantially homogenous (page 20 line 31 to page 21 line 2), biologically functional (page 10, lines

27-28) and activated (page 7, lines 8-9) IKK protein complex comprising transforming a yeast with an IKK subunit gamma (γ) gene and an IKK subunit alpha (α) gene and an IKK subunit beta (β) gene and (page 6, lines 27-29) growing said yeast (page 6, line 30) and separating said IKK protein complex from said yeast (page 7, line 1) thereby preparing substantially homogenous, biologically functional and activated IKK protein complex.

Independent claim 42 is directed to a method for preparing substantially homogenous (page 20 line 31 to page 21 line 2), biologically functional (page 10, lines 27-28) and activated (page 7, lines 8-9) IKK protein complex comprising transforming a yeast with an IKK subunit gamma (γ) gene and an IKK subunit alpha (α) gene and an IKK subunit beta (β) gene (page 6, lines 27-29) and growing said yeast (page 6, line 30) and separating said IKK protein complex from said yeast(page 7, line 1), wherein the IKK protein complex is autophosphorylated at a T loop of an IKK subunit beta (β) (page 7, lines 6-9) thereby preparing substantially homogenous, biologically functional and activated IKK protein complex.

Claim 5, dependent on claim 2 or 42, is directed to inclusion of a tag (page 6, line 28) to the IKK gene sequences.

Claim 6, dependent on claim 2 or 42, is directed to defining the tag to be selected from selected from the group consisting of myc, HA, FLAG and 6his (page 22, Table 1).

Claim 7, dependent on claim 2 or 42, is directed to inclusion of an inducible promoter or a constitutive promoter (page 12, lines 9-12) to the IKK gene sequences.

Claim 17, dependent on claim 2 or 42, is directed to defining the yeast as *Saccharomyces cerevisiae* (page 12, line 1).

Claim 18, dependent on claim 2 or 42, is directed to defining one or more of the IKK gene being a mammalian IKK gene (page 8, line 10 and the originally filed claim 18).

Claim 19, dependent on claim 2 or 42, is directed to defining one or more of the IKK gene being a human IKK gene (page 8, line 10).

Claim 21, dependent on claim 2 or 42, is directed to defining that the yeast is grown in selective liquid media (page 6, line 30).

Claim 22, dependent on claim 2 or 42, is directed to defining that one or more of the IKK gene encodes a wild-type IKK subunit protein (page 10, line 4).

Claim 23, dependent on claim 2 or 42, is directed to defining that one or more of the IKK gene encodes a mutated IKK subunit protein (page 9, line 5-21).

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The claims in this application stand rejected as follows:

Claims 2, 5-7, 17-19, 21-23 and 42 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over Rothwarf *et al.* (1998) *Nature* 395:297-300 (hereinafter "Rothwarf") in view of Traincard *et al.* (1999) *J. Cell Science* 112:3529-35 (hereinafter "Traincard") and Epinat *et al.* (1997) Yeast 16:599-612 (hereinafter "Epinat").

Briefly and for the sake of completeness, the Office alleged that Rothwarf discloses that NIK and MEKK1 phosphorylate IKK *in vitro* (*i.e.*, in the absence of any cellular context) and therefore it is obvious that NIK and MEKK1 can phosphorylate IKK in yeast. Additionally, the Office alleged that Traincard discloses that yeast does not have homologs of any member of the NF-κB signaling system. Further, the Office alleged that Epinat discloses that yeast is a convenient host for reconstitution of the NF-κB system since it does not contain any endogenous NF-κB activity.

ARGUMENT

In general, the claimed invention is directed to preparing an activated IKK protein complex in yeast by transforming yeast with sequences encoding the three subunits of the IKK protein complex - IKKα, IKKβ, and IKKγ. The claimed invention is based on the unexpected finding that IKKγ regulates autophosphorylation of IKKβ leading to self-activation of the IKK complex.

As provided above, it was believed as of the effective filing date of the present application, that activation of the IKK complex requires the TNF-α and NF-κB signaling pathways, such as proteins that activate NIK or MEKK1, and NEMO, a protein that binds and keeps IKK in a condition suitable for activation. Yeast, however, as the Office recognized, lacks the TNF-α and NF-κB signaling pathways or their equivalents. Therefore, it would not have been expected that an activated IKK protein complex can be prepared by transforming yeast with sequences encoding the IKKα, IKKβ, and IKKγ subunits of the IKK protein complex, as the claimed invention prescribes.

In the Final Office Action mailed September 2, 2009, the Office rejected all pending claims (2, 5-7, 17-19, 21-23 and 42) as allegedly obvious over Rothwarf in view of Traincard and Epinat. The Office alleged that Rothwarf teaches that IKK complex can be phosphorylated by NIK and MEKK1 *in vitro* to produce an active IKK complex. The Office further alleged that *in vitro* means in the absence of any cellular context, which in essence implies that activation of IKK by NIK and MEKK1 does not require the presence of the TNF-α and NF-κB signaling pathways. Therefore, the Office argued that IKK complex can be phosphorylated by NIK and MEKK1 in yeast to produce an active IKK complex, even though yeast lacks the TNF-α and NF-κB signaling pathways. Accordingly, the Office argued, the claimed invention directed to generating an active IKK complex in yeast is obvious over the prior art.

Appellants respectfully traverse for the reasons which follow.

(1) Rothwarf does not teach that NIK or MEKK1 phosphorylates IKK in the absence of any cellular context

The Office alleged that Rothwarf discloses that NIK and MEKK1 phosphorylated IKK "*in vitro* (i.e., in the absence of any cellular context)." Office Action mailed September 2, 2009, lines 1 to 4 (emphasis added). Appellants respectfully disagree.

A. *In vitro* does not mean "in the absence of any cellular context"

According to the online Merriam-Webster dictionary, the term "*in vitro*" means "outside the living body and in an artificial environment" http://www.merriam-webster.com/, last accessed January 20, 2010. The term "*in vitro*", as commonly understood by the skilled artisan, does not imply a lack of cellular context, and is actually most commonly used to refer to a biological reaction being carried out in isolated cells, cell lines or cell extracts.

B. The *in vitro* experiments disclosed in Rothwarf were not carried out in the absence of any cellular context

Subsequently, in the Advisory Action mailed December 29, 2009, the Office acknowledged that the term "*in vitro*" does not necessarily mean "in the absence of any cellular context." The Office, however, argued that, in the specific instance, the *in vitro* experiment was carried out in the absence of any cellular context. Appellants respectfully disagree for the reasons that follow.

I. Rothwarf discloses that NIK was expressed and activated in human cells before NIK phosphorylated IKK

As the Office noted in Office Action mailed September 2, 2009, Rothwarf cites Ling *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* **95**:3792-7 (hereinafter "Ling") to support the statement that NIK phosphorylated IKK *in vitro*. Ling, however, discloses that both NIK and IKK proteins were first expressed in 293 cells, a human cell line, and then isolated from the cells before they were incubated together for the kinase assay. *See, e.g.*, page 3793, 1st column, first full paragraph and FIG. 1 legend, and page 3794, FIG.

3 legend. Ling further teaches that NIK becomes activated before phosphorylating IKK. *Id.* page 3797, first full paragraph. Therefore, Ling suggests that NIK would not be able to phosphorylate IKK without NIK first being activated in the human cells by components of the TNF-α and NF-κB signaling pathways. Because activation of NIK in the human cells is a prerequisite for NIK to phosphorylate IKK, the biochemical process of NIK phosphorylating IKK, taken as a whole, does not occur in the absence of any cellular context.

II. Prior art further teaches that activation of IKK by NIK requires IKK to be in a condition suitable for activation

It was known, at the effective filing date of the present application, that activation of IKK by NIK not only requires NIK to be activated first, but also requires IKK to be in a condition suitable for activation.

NF-κB Essential Modulator (NEMO) is a protein that binds to IKK. U.S. Patent No. 6,864,355, hereinafter the "'355 patent", column 24, lines 53 to 63. In the absence of NEMO, IKK is phosphorylated at a serine-rich region of the C-terminus of IKKβ making it refractory to NIK activation. *Id.* column 25, lines 43 to 49. Therefore, the '355 patent teaches that in order for NIK to activate IKK, NEMO needs to be present when IKK is expressed to prevent IKK from being phosphorylated at the serine-rich region of the C-terminus of IKKβ. NEMO, however, like other components of the TNF-α and NF-κB signaling pathways, is not present in yeast.

Accordingly, it would be readily appreciated by the skilled artisan that a IKK protein complex expressed in yeast could not be activated by NIK.

III. Rothwarf discloses that activation of IKK by MEKK1 was carried out in human cell extracts

The Office further alleged that Rothwarf cites Nakano *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* **95**:3537-42 (hereinafter "Nakano") to support the statement that

MEKK1 phosphorylated IKK *in vitro*, and argued that the phosphorylation occurred in the absence of any cellular context. Appellants respectfully disagree.

Appellants submit that Nakano cites Lee *et al.*, (1997) *Cell* 88:213-22 (hereinafter "Lee") to support the statement that MEKK1 phosphorylated IKK *in vitro*. Lee, page 3541, 2nd column, last paragraph. Lee, however, discloses that the *in vitro* phosphorylation of IκBα kinase, an IKK protein, by MEKK1 was observed in cytoplasmic extracts of HeLa cells. *See, e.g.*, page 216, 2nd column, first full paragraph. Because Hela is a human cell line, it is clear that the phosphorylation of IKK by MEKK1 was in the presence of cellular context, rather than in the absence of cellular context, as the Office alleged.

Appellants further submit that, compared to NIK, the molecular mechanisms by which MEKK1 is activated and MEKK1 activates IKK were even less well understood at the time the application was filed. It was even suspected that MEKK1 does not directly activate IKK. *See, e.g.*, Karin *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:9067-9, at page 9067, second column, second to the last sentence, and Figure 1. Therefore, the teachings of Rothwarf, Nakano or Lee relating to IKK's phosphosphoration by MEKK1 in human cell extracts do not teach or suggest that MEKK1 can phosphorylate IKK in the absence of any cellular context, or in yeast which lacks the TNF-α and NF-κB signaling pathways.

Because the prior art references do not teach that IKK complex can be phosphorylated and activated by NIK or MEKK1 in the absence of the TNF-α and the NF-κB signaling pathways which yeast lacks, it is not obvious to generate an activated IKK complex in yeast, as prescribed by the claimed invention.

(2) The prior art does not teach or suggest preparation of an autophosphorylated and activated IKK as prescribed by claim 42

In the response filed July 28, 2009, claim 42 was amended to recite that the IKK protein complex is autophosphorylated at a T loop of the IKK subunit beta (β).

Response filed July 28, 2009, page 3. Arguments were presented to support that, in spite of the Office's erroneous interpretation of the teachings of the Rothwarf, claim 42 as well as claims partially dependent from claim 42, namely claims 5-7, 17-19 and 21-23, is not obvious in view of the prior art. *Id.* at section 4 on page 12. The Office, though not raising objection to the amendment, failed to consider (or, at least, comment on) the amendment or the arguments, as required by *MPEP § 2145.*

Appellants note that, in the Final Office Action mailed June 9, 2009, the Office cited a '355 patent. Although not in the form of a rejection, because the '355 patent relates to the merit of claim 42, the arguments in the Office Action relating to the '355 patent are further discussed and responded to as follows.

Briefly and for the sake of completeness, the Office alleged that the '355 patent discloses that IKK-β is autophosphorylated at a serine-rich region of the C-terminus. Appellants submit, however, that this autophosphorylation serves to down-regulate IKKβ activity and make IKKβ refractory to activation rather than to activate IKK, as prescribed by claim 42 of the present application. The '355 patent, at column 25, lines 40 to 48.

Finally, the Office failed to consider, or at least to comment on, the additional limitations of the claimed invention as recited in the dependent claims. For example, the Office failed to cite prior art reference disclosing that each IKK subunit protein is a mutated protein (claim 23). Claim 23, therefore, is nonobvious in light of the cited prior art.

The most recent Office Actions including the Final Office Action did not address the patentability of the dependent claims 5-7, 17-19 and 21-23. The last Action to address the additional limitations of the dependent claims was the Final Office Action mailed February 6, 2008. For the sake of completeness and to be clear that Applicant do not acquiesce in the rejection of these claims, Appellants note the failure of the cited references to teach or suggest the claimed inventions.

Conclusions

The prior art references cited by the Office do not teach that IKK protein complex can be activated in the absence of any cellular context as alleged by the Office. In the contrary, the prior art and other scientific literatures teach that IKK can not be activated without the presence of the TNF-α and the NF-κB signaling pathways which yeast lacks. The teachings of the prior art, therefore, do not render obvious that activated IKK protein complex can be generated from yeast, as prescribed by claims 2, 5-7, 17-19, 21-23 and 42.

Further, because the prior art and the '355 patent cited by the Office do not teach a IKK autophosphorylation that activates IKK, they do not render obvious that activated and autophosphorylated IKK protein complex can be generated from yeast, as prescribed by claims 42 the partially claims 5-7, 17-19 and 21-23 that depend from claim 42.

Accordingly, Appellant respectfully requests withdrawal of the rejection.

CLAIMS APPENDIX

1. (Canceled)

2. (Previously Presented) A method for preparing substantially homogenous, biologically functional and activated IKK protein complex comprising transforming a yeast with an IKK subunit gamma (γ) gene and an IKK subunit alpha (α) gene and an IKK subunit beta (β) gene and growing said yeast and separating said IKK protein complex from said yeast thereby preparing substantially homogenous, biologically functional and activated IKK protein complex.

3. (Canceled)

4. (Canceled)

5. (Previously Presented) The method of claim 2 or 42, wherein one or more of said IKK subunit (γ) gene, or IKK subunit (α) gene or IKK subunit (β) gene further comprises a sequence encoding a tag.

6. (Previously Presented) The method of claim 5, wherein said tag is selected from the group consisting of myc, HA, FLAG and 6his.

7. (Previously Presented) The method of claim 2 or 42, wherein one or more of said IKK subunit (γ) gene, or IKK subunit (α) gene or IKK subunit (β) gene is linked to an inducible promoter or a constitutive promoter.

Claims 8 –16. (Canceled).

17. (Previously Presented) The method of claim 2 or 42, wherein said yeast is Saccharomyces cerevisiae.

18. (Previously Presented) The method of claim 2 or 42, wherein one or more of said IKK subunit (γ) gene, or IKK subunit (α) gene or IKK subunit (β) gene is a mammalian IKK gene.

19. (Previously Presented) The method of claim 18, wherein one or more of said mammalian IKK subunit (γ) gene, or mammalian IKK subunit (α) gene or mammalian IKK subunit (β) gene is a human.

20. (Canceled)

21. (Previously Presented) The method of claim 2 or 42, wherein said yeast is grown in selective liquid media.

22. (Previously Presented) The method of claim 2 or 42, wherein one or more of said IKK subunit (γ) gene, or IKK subunit (α) gene or IKK subunit (β) gene encodes a wild-type IKK subunit protein.

23. (Previously Presented) The method of claim 2 or 42, wherein one or more of said IKK subunit (γ) gene, or IKK subunit (α) gene or IKK subunit (β) gene encodes a mutated IKK subunit protein.

Claims 24 – 41. (Canceled)

42. (Previously Presented) A method for preparing substantially homogenous, biologically functional and activated IKK protein complex comprising transforming a

yeast with an IKK subunit gamma (γ) gene and an IKK subunit alpha (α) gene and an IKK subunit beta (β) gene and growing said yeast and separating said IKK protein complex from said yeast, wherein the IKK protein complex is autophosphorylated at a T loop of an IKK subunit beta (β) thereby preparing substantially homogenous, biologically functional and activated IKK protein complex.

EVIDENCE APPENDIX

The following evidence was introduced and entered into the record during prosecution, and are presently cited by the Office against the claims or by the Appellants to support the arguments.

1. Rothwarf *et al.* (1998) *Nature* 395:297-300. Entered the record when cited by Examiner in the Office Action mailed December 29, 2004 and was originally submitted by Appellants as reference C27 in Information Disclosure Statement filed July 1, 2002.

2. Traincard *et al.* (1999) *J. Cell Science* 112:3529-35. Entered the record when cited by Examiner in the Office Action mailed April 21, 2006.

3. Epinat *et al.* (1997) Yeast 13:599-612. Entered the record when cited by Examiner in the Office Action mailed December 29, 2004.

4. Ling *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* **95**:3792-7. Entered the record when cited by Examiner in the Office Action mailed September 2, 2009 and was originally submitted by Appellants as reference C56 in Information Disclosure Statement filed July 28, 2009.

5. U.S. Patent No. 6,864,355. Entered the record when cited by Examiner in the Office Action mailed June 9, 2009.

6. Nakano *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* **95**:3537-42. Entered the record when cited by Examiner in the Office Action mailed September 2, 2009.

7. Lee *et al.*, (1997) *Cell* 88:213-22. Cited and relied upon by Nakano, which entered the record when cited by Examiner in the Office Action mailed September 2, 2009. Submitted as Exhibit A in the Amendment and Reply filed December 1, 2009.

8. Karin *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 95:9067-9. Submitted in by Appellants as reference C52 in Information Disclosure Statement filed July 17, 2008, and was considered by the Office as indicated in the Office Action mailed October 15, 2008.

RELATED PROCEEDINGS APPENDIX

As indicated above, Appellant is unaware of any other prior or pending appeals, interferences or judicial proceedings which may be related to, directly affect or be directly affected by or have a bearing on the decision in this application. Accordingly, no decision has been rendered by a court or the Board in a related proceeding.

Respectfully submitted,

Date: April 5, 2010

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IKK-γ is an essential regulatory subunit of the IκB kinase complex

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Pro-inflammatory cytokines activate the transcription factor NF-κB by stimulating the activity of a protein kinase that phosphorylates IκB, an inhibitor of NF-κB[1–5], at sites that trigger its ubiquitination and degradation. This results in the nuclear translocation of freed NF-κB dimers and the activation of transcription of target genes[6,7]. Many of these target genes code for immunoregulatory proteins[8,9]. A large, cytokine-responsive IκB kinase (IKK) complex has been purified and the genes encoding two of its subunits have been cloned[1,2,5]. These subunits, IKK-α and IKK-β, are protein kinases whose function is needed for NF-κB activation by pro-inflammatory stimuli. Here, by using a monoclonal antibody against IKK-α, we purify the IKK complex to homogeneity from human cell lines. We find that IKK is composed of similar amounts of IKK-α, IKK-β and two other polypeptides, for which we obtained partial sequences. These polypeptides are differentially processed forms of a third subunit, IKK-γ. Molecular cloning and sequencing indicate that IKK-γ is composed of several potential coiled-coil motifs. IKK-γ interacts preferentially with IKK-β and is required for the activation of the IKK complex. An IKK-γ carboxy-terminal truncation mutant that still binds IKK-β blocks the activation of IKK and NF-κB.

We previously purified the IKK complex from HeLa cells treated with tumour-necrosis factor (TNF)[1]. The complex contains two catalytic subunits, IKK-α and IKK-β, of relative molecular mass 85,000 and 87,000 (M_r 85K and 87K), respectively. These subunits have a kinase domain in their amino-terminal portion and protein-interaction motifs, including a leucine zipper and a helix–loop–helix (HLH)[1–3], in their C-terminal region[1,2]. IKK-α and IKK-β are rapidly activated by TNF and interleukin-1 (IL-1) and are necessary for NF-κB activation[1,2,5]. IKK activity depends on its phosphorylation, as it is inactivated by protein phosphatase 2A (ref. 1). The exact subunit whose dephosphorylation causes loss of IKK activity is not yet known. IKK-α/β can be phosphorylated and activated by over-expressed NF-κB-inducing kinase (NIK)[10] or by MEK kinase-1 (MEKK-1)[11], but the physiological role of NIK and MEKK-1 in IKK activation by pro-inflammatory cytokines is not clear[12]. The composition of the IKK complex and the function of its various subunits needed to be determined.

To purify IKK to homogeneity, we prepared monoclonal antibodies specific for IKK-α; one of these antibodies is very efficient in precipitating IKK activity. Using it as an immunoaffinity reagent, we found that, in both HeLa and Jurkat cells, the IKK complex contained nearly equal amounts of IKK-α and IKK-β (Fig. 1). Furthermore, passing partially purified IKK fractions through the anti-IKKα column resulted in quantitative recovery of IKK-β (Fig. 1a), with which this antibody does not crossreact (our unpublished results). Large-scale purification of IKK, by combining the previous method[1] with immunoaffinity chromatography on immobilized anti-IKKα, showed that, in addition to IKK-α/β, purified IKK contained two polypeptides of M_r 50K and 52K (Fig. 1b). Similar results were obtained by immunoprecipitation

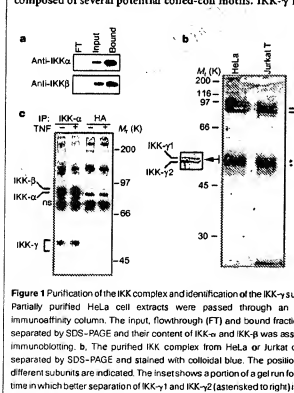

Figure 1 Purification of the IKK complex and identification of the IKK-γ subunits. **a**, Partially purified HeLa cell extracts were passed through an anti-IKKα immunoaffinity column. The input, flowthrough (FT) and bound fractions were separated by SDS–PAGE and their content of IKK-α and IKK-β was assessed by immunoblotting. **b**, The purified IKK complex from HeLa or Jurkat cells was separated by SDS–PAGE and stained with colloidal blue. The positions of the different subunits are indicated. The inset shows a portion of a gel run for a longer time in which better separation of IKK-γ1 and IKK-γ2 (asterisked to right) is seen. **c**, 293 cells were labelled with ^{35}S for 5 h, followed by incubation with or without TNF. Lysates were immunoprecipitated (IP) with either anti-IKKα or anti-HA (used as a control) antibodies. After extensive washing the immune complexes were separated by SDS–PAGE and visualized by autoradiography. ns, nonspecific band.

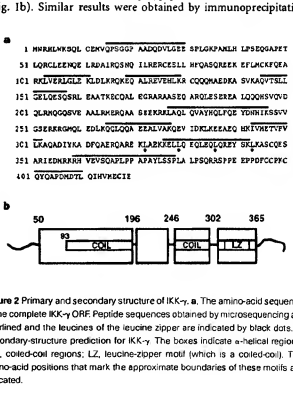

Figure 2 Primary and secondary structure of IKK-γ. **a**, The amino-acid sequence of the complete IKK-γ ORF. Peptide sequences obtained by microsequencing are overlined and the leucines of the leucine zipper are indicated by black dots. **b**, Secondary-structure prediction for IKK-γ. The boxes indicate α-helical regions. Coil, coiled-coil regions; LZ, leucine-zipper motif (which is a coiled-coil). The amino-acid positions that mark the approximate boundaries of these motifs are indicated.

of ^{35}S-labelled cell lysates (Fig. 1c). TNF treatment did not change the relative amounts of these polypeptides. Microsequencing of these polypeptides indicated that they are either differentially processed or differentially initiated forms of the same protein (see Methods), which we named IKK-γ. Screening of Genebank for sequences that are identical or similar to these peptides revealed two overlapping expressed sequence tags (ESTs). A complementary DNA probe corresponding to these ESTs was used to isolate a cDNA clone containing the entire IKK-γ open reading frame (ORF) (Fig. 2a). The predicted IKK-γ polypeptide is 419 amino acids long. Sequence analysis shows that IKK-γ is a new protein and is glutamine-rich. Programs for secondary-structure prediction[13–15] indicate that the prominent features of IKK-γ are two extended coiled-coil motifs and a leucine zipper (Fig. 2b). Such motifs are likely to be involved in protein–protein interactions.

To confirm that the cloned IKK-γ protein interacts with IKK-α/β subunits in cells, we transfected an expression vector for N-terminally haemagglutinin (HA)-tagged IKK-γ into HeLa cells, together with expression vectors for either Flag-tagged IKK-α or Flag-tagged IKK-β. Transfected cell lysates were immunoprecipitated with anti-HA antibody and then immunoblotted with anti-Flag antibody. This analysis confirmed that IKK-γ interacts efficiently with either IKK-α or IKK-β (Fig. 3a). Similar results were obtained when the immunoprecipitating antibody was directed to the Flag epitope and the immune complexes were immunoblotted with anti-HA antibody, which recognizes the epitope on IKK-γ. Immunoprecipitation of transiently expressed HA–IKK-γ resulted in isolation of endogenous IKK-α (Fig. 3b) and immunoprecipitation of endogenous IKK-α co-precipitated HA–IKK-γ (Fig. 3c). Because of a lack of high-affinity antibodies for IKK-β, we were unable to study the interaction between HA–IKK-γ and endogenous IKK-β. The interaction between IKK-γ and IKK-α was not altered by cytokines (Figs 1c and 3b).

As IKK-α and IKK-β form very stable heterodimers[16], these results do not indicate whether IKK-γ binds directly to IKK-α, IKK-β or both proteins. We therefore studied interactions of IKK-γ with IKK-α or IKK-β using purified recombinant proteins (Fig. 3d). We detected direct and stable binding of IKK-γ to IKK-β but not to IKK-α.

Immunoprecipitation of HA–IKK-γ from transiently transfected cells resulted in isolation of an IκB kinase activity that was stimulated by either TNF or IL-1 (Fig. 4a). These results were identical to those obtained when anti-IKKα was used to isolate the IKK complex. This similarity is due to efficient interaction between transiently expressed HA–IKK-γ and other IKK components. Gel-filtration analysis indicated that HA–IKK-γ was incorporated into the large, 900K IKK complex, precisely co-eluting with IKK-α (Fig. 4b).

We first studied the involvement of IKK-γ in IKK activation through expression of IKK-γ antisense RNA. Whereas co-transfection of an IKK-γ sense vector had no effect on IκB kinase activity associated with either IKK-α or IKK-β (see below), the level of TNF-induced IκB kinase activity associated with either IKK subunit decreased upon co-transfection with the IKK-γ antisense vector (Fig. 5a). Antisense IKK-γ reduced the expression of HA–IKK-γ, but had no effect on the expression of either HA–IKKα or HA–IKKβ (Fig. 5b). A cDNA that can complement IKK activity in two variant cell lines that are defective in NF-κB activation has recently been identified[11]. The product of this cDNA, named NF-κB essential modulator (NEMO), seemed to be the mouse homologue of IKK-γ.

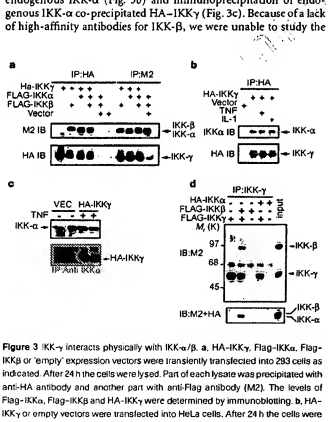

Figure 3 IKK-γ interacts physically with IKK-α/β. **a**, HA–IKK-γ, Flag–IKKα, Flag–IKKβ or 'empty' expression vectors were transiently transfected into 293 cells as indicated. After 24 h the cells were lysed. Part of each lysate was precipitated with anti-HA antibody and another part with anti-Flag antibody (M2). The levels of Flag–IKKα, Flag–IKKβ and HA–IKK-γ were determined by immunoblotting. **b**, HA–IKK-γ or empty vectors were transfected into HeLa cells. After 24 h the cells were left untreated or incubated with either TNF or IL-1, lysed, immunoprecipitated with anti-HA antibody and immunoblotted with anti-IKKα and anti-HA antibody. **c**, HA–IKK-γ or empty (VEC) vectors were transfected into 293 cells, which were treated and processed as in **b**. **d**, HA–IKKα and Flag–IKKβ were expressed in Sf9 cells using baculovirus vectors and were purified[16]. They were incubated with or without purified recombinant Flag–IKK-γ. The proteins were immunoprecipitated with anti-IKK-γ (anti-NEMO)[17] antibody and immunoblotted with anti-HA and anti-Flag (M2) antibody. IP, immunoprecipitation; IB, immunoblot.

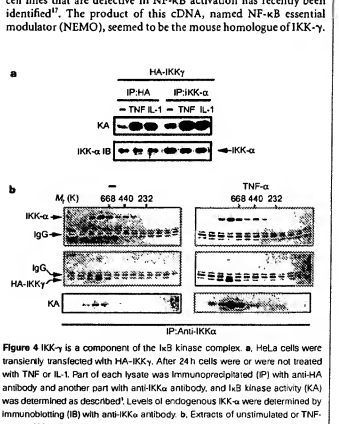

Figure 4 IKK-γ is a component of the IκB kinase complex. **a**, HeLa cells were transiently transfected with HA–IKK-γ. After 24 h cells were or were not treated with TNF or IL-1. Part of each lysate was immunoprecipitated (IP) with anti-HA antibody and another part with anti-IKKα antibody, and IκB kinase activity (KA) was determined as described[1]. Levels of endogenous IKK-α were determined by immunoblotting (IB) with anti-IKKα antibody. **b**, Extracts of unstimulated or TNF-treated 293 cells that were transfected with an HA–IKK-γ vector were fractionated on a Superose 6 column. Fractions were immunoprecipitated with anti-IKKα antibody. Immune complex kinase assays (KA) and immunoblotting of IKK-α and HA–IKK-γ were done with IKK-α and HA antibodies.

Using anti-NEMO antibodies (a gift from A. Israel), we confirmed this identity and found that transient expression of antisense IKK-γ RNA reduced expression of endogenous IKK-γ but not of IKK-α (Fig. 5c) or IKK-β (data not shown).

Antisense IKK-γ also reduced the extent of IKK activation by IL-1 or transiently transfected MEKK-1 or NIK vectors, although the inhibition of the response to overexpressed NIK was considerably less than that of the other responses (Fig. 5d). Co-transfection of antisense vectors for the kinases JNKK-1 and MKK-3 had no effect on IKK activity[1] and antisense IKK-γ did not inhibit the activation of the kinase $p38^{MAPK}$ by either TNF or IL-1 (Fig. 6e). Transient expression of antisense IKK-γ prevented TNF-induced nuclear entry of the RelA (p65) subunit of NF-κB (data not shown). We also established stably transfected pools of 293 cells containing the antisense IKK-γ expression vector. Cells in these pools expressed less IKK-γ (Fig. 7b) and, compared with the parental cells, exhibited less TNF-induced IκB-α phosphorylation and degradation (Fig. 7a) and NF-κB activation (Fig. 7c). This inhibitory effect was specific as IKK-α expression was not decreased (Fig. 7a) and the DNA-binding activity of the constitutive transcription factor NF-1 was actually increased in cells transfected with antisense IKK-γ (Fig. 7c). In addition, the antisense-IKKγ-transfected cells exhibited normal activation of the kinases JNK and $p38^{MAPK}$ (Fig. 7d).

To further study the function of IKK-γ and gain clues as to how it is involved in regulation of IKK activity, we constructed N- and C-terminal deletion mutants (Fig. 6a) and studied them for possible dominant inhibitory activity. Expression of ΔN-IKKγ(134–419) with Flag–IKKβ had only a marginal effect on basal IKK activity and its response to TNF. However, expression of ΔC-IKKγ(1–300) inhibited activation of IKK by TNF but not basal kinase activity (Fig. 6b). Both ΔN-IKKγ(134–419) and ΔC-IKKγ(1–300) interacted with IKK-α/β in cells (Fig. 6c), but only full-length IKKγ and ΔN-IKKγ(134–419) were able to co-precipitate IKK activity stimulated by TNF, IL-1, MEKK-1 or NIK (data not shown). Cross-linking experiments using recombinant proteins indicated that IKK-γ can form dimers and trimers and that the C-terminal truncation had no effect on this activity, although the N-terminal truncation may have reduced the efficiency of trimerization. Neither full-length IKK-γ nor its truncation mutants inhibited activation of $p38^{MAPK}$ by TNF or IL-1 (Fig. 6e).

Previous studies showed that IKK contains two catalytic subunits, IKK-α and IKK-β[2,4,5]. The identity of the remaining IKK subunits and their function were unknown until now. We have purified IKK to homogeneity and found that, in addition to IKK-α/β, it contains two other major polypeptides, IKK-γ1 and IKK-γ2, which are derived from the same transcript. The presence of leucine zippers and coiled-coil motifs indicates that IKK-γ can be involved in homotypic and heterotypic interactions. Indeed, recombinant IKK-γ forms dimers and trimers and can directly interact with IKK-β. As IKK-α and IKK-β form stable homodimers and heterodimers even in the absence of IKK-γ[16], the core IKK complex might consist of an IKKα–IKKβ heterodimer associated with an IKK-γ dimer or trimer.

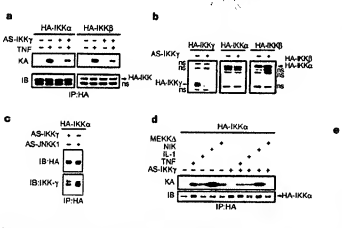

Figure 5 IKK-γ is an essential component of the IκB kinase. **a**, HA–IKKα and HA–IKKβ vectors were transfected into HeLa cells together with either 'empty' or antisense (AS)-IKKγ vectors. After 24 h cells were or were not treated with TNF and were lysed. Lysates were precipitated with anti-HA antibody and IKK activity was determined by immune complex kinase assays (KA). Expression of HA–IKKα and HA–IKKβ was determined by immunoblotting. Migration positions of IKKα and IKK-β and of a nonspecific (ns) band are indicated. **b**, HA–IKKγ, HA–IKKα or HA–IKKβ vectors were transfected into 293 cells together with either 'empty' or AS–IKKγ vectors as indicated. After 24 h the cells were lysed, immunoprecipitated with anti-HA antibody and immunoblotted with anti-HA antibody. **c**, HeLa cells were co-transfected with HA–IKKα and either AS–IKKγ or AS–JNKK1 vectors. After 24 h cell lysates were prepared, immunoprecipitated with anti-HA antibody and immunoblotted with anti-HA antibody or anti-NEMO (anti-IKKγ) antibody. **d**, HeLa cells were transfected with HA–IKKα and either 'empty' or AS–IKKγ vectors, together with NIK or MEKK-1 catalytic domain (MEKKΔ) expression vectors, as indicated. After 24 h cells were or were not treated with TNF or IL-1 and were lysed and immunoprecipitated with anti-HA antibody. IKK activity was determined as above. IP, immunoprecipitation; IB, immunoblot.

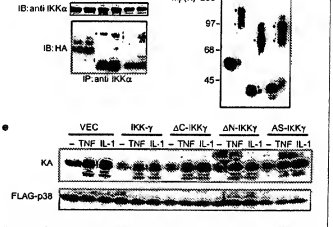

Figure 6 A C-terminal IKK-γ deletion mutant is a dominant-negative inhibitor of IKK activation. **a**, Full-length (FL) IKK-γ and its deletion mutants. **b**, Flag–IKKβ was transfected together with wild-type and truncated IKK-γ expression vectors into HeLa cells. After 24 h cells were or were not incubated with TNF and were then lysed. The lysates were immunoprecipitated with anti-Flag antibody (M2), and IKK kinase activity (KA) and Flag–IKKβ expression were determined. **c**, 293 cells were transfected with the different HA–IKKγ vectors and treated as above. Lysates were immunoprecipitated with anti-IKKα antibody and immunoblotted (IB) with anti-IKKα and anti-HA antibodies. **d**, Recombinant full-length IKK-γ and its truncation mutants were expressed in *E. coli*, purified and incubated with or without the crosslinking agent ethylene glycolbis(succinimidylsuccinate) (EGS). The proteins were separated by SDS–PAGE and visualized by immunoblotting. The asterisks and dots mark the dimer and trimer bands, respectively. **e**, Flag–p38 vector was transfected together with either 'empty' vector (VEC) or IKK-γ sense and antisense (AS) expression vectors. After 24 h the cells were either left untreated or were incubated with TNF or IL-1. Lysates were prepared, and p38 activity and expression were determined by immune complex kinase assay (KA) and immunoblotting, respectively.

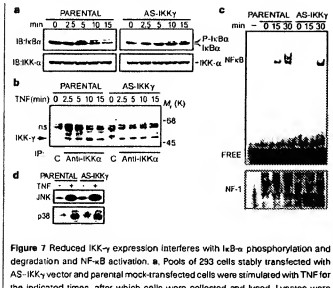

Figure 7 Reduced IKK-γ expression interferes with IκB-α phosphorylation and degradation and NF-κB activation. **a**, Pools of 293 cells stably transfected with AS-IKK-γ vector and parental mock-transfected cells were stimulated with TNF for the indicated times, after which cells were collected and lysed. Lysates were tested by immunoblotting for IκBα degradation and endogenous IKK-α levels. Basal and phosphorylated (P-IκBα) forms of IκBα are indicated. **b**, IKK-α immune complexes were isolated and immunoblotted with anti-NEMO (anti-IKK-γ) antibody. C, immunoprecipitation with control antibody; ns, nonspecific band. **c**, Parental cells or pools of 293 cells stably transfected with the AS-IKK-γ vector were incubated with TNF for the indicated times, after which nuclear extracts were prepared. The levels of DNA-binding activities of NF-κB and NF-1 were determined by electrophoretic mobility shift assay[18]. **d**, JNK and p38 activities were determined by immune complex kinase assay IP, immunoprecipitation; IB, immunoblot.

Although the exact biochemical function of IKK-γ is yet to be determined, it is an essential component of IKK. Reduced IKK-γ expression results in decreased IKK activation (Figs 5 and 7) and its complete absence abolishes IKK and NF-κB activation altogether[17]. Although IKK activity is absolutely dependent on IKK-α/β dimerization[3], IKK-γ is unlikely to function as a chaperone or a co-factor that stabilizes IKKα–IKKβ dimers. The ability of the C-terminally truncated IKK-γ mutant to inhibit IKK activation by upstream stimuli, while having only a small effect on basal kinase activity, indicates that the major function of IKK-γ may be to connect the IKK complex to upstream activators. This function is likely to be mediated by the C-terminal region of IKK-γ, while its central region probably interacts with IKK-β. Although, *in vitro*, IKK-γ stably interacts with IKK-β and not with IKK-α, it is likely that once recruited into the complex IKK-γ also interacts with IKK-α. □

Methods

Purification and cloning of IKK-γ. The IKK complex was purified from HeLa and Jurkat cells as described[1] except for substituting affinity chromatography on an IκBα(1–54) column with affinity chromatography on immobilized monoclonal anti-IKKα antibody (B78–743) (this antibody is avilable from PharMingen), generated against full-length recombinant IKK-α. Purified antibody (0.5 mg) was coupled to 0.3 ml CNBr-activated Sepharose 4B (Pharmacia). Active IKK fractions, after gel filtration on Superose 6, were pooled (1.6 ml) and applied batchwise to the immunoaffinity resin (0.1 ml). The mixture was rotated at 4 °C for 4 h and then centrifuged. The beads were washed with 20 ml buffer A[1] containing 500 mM NaCl and 1% Triton X-100 and then with 5 ml buffer A containing 2 M urea. Bound protein was eluted with 0.5% SDS and separated by SDS–PAGE. Bands identified as IKK-γ were transferred to a PVDF membrane, stained with colloidal blue and digested with Lys-C. The IKK-γ1 band was also obtained in pure form after gel filtration and SDS–PAGE without requiring the immunoaffinity step. This material was transferred to a PVDF membrane and digested with Lys-C. A total of 15 peptide sequences derived from the 52K (IKK-γ1) and 50K (IKK-γ2) forms was obtained (by T. W. Thannhauser, data not shown). All of these sequences are contained within the IKK-γ ORF (Fig. 2). The peptide maps generated by Lys-C digestion of IKK-γ1 and IKK-γ2 are very similar (data not shown). These sequence data were used to search Genebank and two overlapping human ESTs were found (accession numbers AA133061 and R56495). Primers derived from these ESTs were used to generate a probe to isolate a clone containing the complete IKK-γ ORF from a HeLa cDNA library. The complete IKK-γ nucleotide sequence is available under accession number AF074382.

Kinase assays, immunoprecipitation, immunoblotting, immunofluorescence and gel-shift assay. Kinase assays, immunoprecipitation, immunoblotting and indirect immunofluorescence experiments were done as described[1,3]. The monoclonal anti-IKKα antibody was used for immunoblotting and immunoprecipitation. This antibody does not crossreact with IKK-β (data not shown). TNF-α and IL-1 were used at 20 ng ml^{-1} and 10 ng ml^{-1}, respectively. Induction times were 10 min except where indicated otherwise (in figure legends). The probes used in the gel-shift assay correspond to the consensus κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and NF-1 (5′-TTGGATTGAAGCCAATATGATA-3′) sites.

Plasmids, cell culture, transfections and ^{35}S labelling. The expression vectors were constructed using standard recombinant DNA procedures. The β-actin promoter was used to drive expression of all sense IKK-γ constructs. Antisense IKK-γ constructs were made in vector pcDNA3.1/Myc-His (Invitrogen). Cell culture and transfections were as described[1] except that Lipofectamine Plus (Gibco) was used. IKK mutants were generated as described[3]. ^{35}S labelling was carried out using Pro-Mix (Amersham).

Expression and purification of recombinant proteins. Recombinant HA–IKKα and Flag–IKKβ were expressed in Sf9 cells using recombinant baculovirus vectors and were purified to homogeneity[3,5]. Recombinant hexahistidine-tagged IKK-γ proteins (full-length and truncation mutants) were expressed in *Escherichia coli* and purified by nickel-affinity chromatography.

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Evidence for the presence of an NF-κB signal transduction system in *Dictyostelium discoideum*

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SUMMARY

The Rel/NF-κB family of transcription factors and regulators has so far only been described in vertebrates and arthropods, where they mediate responses to many extracellular signals. No counterparts of genes coding for such proteins have been identified in the *Caenorhabditis elegans* genome and no NF-κB activity was found in *Saccharomyces cerevisiae*. We describe here the presence of an NF-κB transduction pathway in the lower eukaryote *Dictyostelium discoideum*. Using antibodies raised against components of the mammalian NF-κB pathway, we demonstrate in *Dictyostelium* cells extracts the presence of proteins homologous to Rel/NF-κB, IκB and IKK components. Using gel-shift experiments in nuclear extracts of developing *Dictyostelium* cells, we demonstrate the presence of proteins binding to κB consensus oligonucleotides and to a GC-rich κB-like sequence, lying in the promoter of *cbpA*, a developmentally regulated *Dictyostelium* gene encoding the Ca^{2+}-binding protein CBP1. Using immunofluorescence, we show specific nuclear translocation of the p65 and p50 homologues of the NF-κB transcription factors as vegetatively growing cells develop to the slug stage. Taken together, our results strongly indicate the presence of a complete NF-κB signal transduction system in *Dictyostelium discoideum* that could be involved in the developmental process.

Key words: *Dictyostelium discoideum*, NF-κB, Signal transduction

INTRODUCTION

In eukaryotes, the Rel/NF-κB family of transcription factors is involved in many cellular processes, including cell proliferation (Bauerle and Henkel, 1994), differentiation (Miyamoto and Verma, 1995), apoptosis (Kasibhatla et al., 1998), and cell responses to injury, stress and external pathogens (for a review see Bauerle and Henkel, 1994). Each of the Rel/NF-κB transcription factors contains a Rel-Homology Domain (RHD), including a DNA-binding site, a nuclear localization sequence, a dimerization domain and amino acids, which are responsible for the interaction with the inhibitory proteins of the IκB family (Bauerle and Henkel, 1994; Miyamoto and Verma, 1995; Siebenlist et al., 1994). In unstimulated cells, IκB proteins bind to Rel/NF-κB transcription factors through ankyrin repeats, which results in masking their nuclear localization sequence (Miyamoto and Verma, 1995) and their sequestration in the cytoplasm. Upon cell stimulation, inhibitors of the IκB family are phosphorylated by IκB kinases (IKK), which allows their ubiquitination and subsequent degradation by the 26S proteasome (for a review see May and Ghosh, 1998). The free Rel/NF-κB transcription factors then translocate into the nucleus, where they bind target promoters containing Rel-recognition sites (Chytil and Verdine, 1996).

Five proteins of the Rel/NF-κB family have been discovered so far in mammals: p65, RelB, p50/p105, p52/p100 and cRel, the cellular counterpart of the viral oncogene *v-Rel*. Related proteins have been identified in lower species, including XRel-1 and XRel-2 in *Xenopus laevis* and Dorsal, Dif and Relish in *Drosophila melanogaster*. In mammals, seven proteins belonging to the IκB family have been found (IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100 and p105) as well as two IκB kinases (IKKα and IKKβ). A member of the IκB family, named cactus, has also been found in *Drosophila*. In contrast, no counterparts of Rel/NF-κB or IκB-coding genes have been identified in the *Caenorhabditis elegans* genome (Ruvkun and Hobert, 1998) nor in *Saccharomyces cerevisiae*, and no NF-κB activity was found in this latter organism (Epinat et al., 1997).

Dictyostelium discoideum is a primitive eukaryote, which feeds on bacteria by phagocytosis. Upon starvation, the amoebae aggregate to form a multicellular structure, which further differentiates into a fruiting body made of a long stalk supporting a mass of spores. Although many genes are involved in this morphogenetic process (for a review, see Firtel, 1996), only very few transcription factors have been identified so far in *Dictyostelium*, including GBF (Schnitzler et al., 1994) and a member of the STAT family (Kawata et al., 1997). These factors were shown to be involved in development controlling, respectively, the developmental transition between aggregation

and multicellular morphogenesis, and acting in the regulation of stalk cell differentiation. Although *Dictyostelium* appears to have diverged early in evolution from the branch leading to higher eukaryotes (Loomis and Smith, 1995), it harbours regulatory pathways present in mammals but absent in some other eukaryotes. STAT, for example, is absent in *C. elegans* (Ruvkun and Hobert, 1998).

Using an immunological approach and electrophoretic mobility-shift assays to search for proteins binding to consensus κB oligonucleotides, we present the first evidence for the presence of an NF-κB pathway in *Dictyostelium*.

MATERIALS AND METHODS

Materials

Antibodies against p65, p52/p100, IκBβ, IKKα and IKKβ were from Santa-Cruz Biotechnology (Santa Cruz, CA) and raised, respectively, against the C-terminal peptide of human p65 (amino acids 537-550), a full-length human p52 protein, a C-terminal peptide of human IκBβ (amino acids 339-358), a full-length human IKKα protein, and a recombinant protein corresponding to amino acids 470-755 mapping at the C terminus of human IKKβ. Antibodies against full-length human p65 (anti-p65$_2$, 39043) were a gift from Dr Christel Brou. Antiserum against amino acids 1-502 of p50/p105 (351) has been described previously (Kieran et al., 1990). Antibodies against full-length human IκBα (52008) and amino acids 258-360 of mouse IκBβ (37015) (Weil et al., 1997) were a kind gift from Dr Robert Weil.

Cell and nuclear extract preparation

Dictyostelium AX2 cells were grown axenically in HL5 broth (Watts and Ashworth, 1970). Development was carried out on 0.9% nonnutrient agar plates (2.5x10^6 cells/cm^2) containing SP buffer (15 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.0). Nuclear extracts from vegetative cells and cells starved for 16 hours on SP agar were prepared as described by Kawata et al. (1997), except that crude nuclear extracts were subjected to a 70% $(NH_4)_2SO_4$ precipitation. Samples were stored at −80°C. Cell extracts for native western blots were prepared as follows. The 70Z/3 murine pre B cells were washed twice in PBS (0.15 M NaCl containing 0.01 M phosphate buffer, pH 7.4) and lysed as described in Weil et al. (1997). *Dictyostelium* cells were washed twice in SP buffer and lysed by two freeze-thaw cycles. *Dictyostelium* and mouse cell lysates were then clarified by centrifugation at 13,000 *g* (15 minutes, 4°C) and supernatants were kept frozen at −80°C.

Western blots

Denaturing western blots were performed on total extracts from vegetative cells as described by Dammann et al. (1998). For native western blots, cell supernatants were diluted (v/v) with 2× non-denaturing loading buffer (pH 8.3). A native polyacrylamide gel (7.5%, pH 8.3) was loaded with 40 μg of protein extract per slot. Membrane transfer and immunoprobing was performed as under denaturing conditions. For both techniques, antibodies were used at a concentration of 200 ng/ml for Santa-Cruz antibodies, or diluted 1/1000 for all other antibodies.

Electrophoretic mobility-shift assays

Fig. 1 shows the sequences of the double-stranded synthetic oligonucleotides used in electrophoretic mobility-shift assay (EMSA) experiments with *Dictyostelium* extracts. Igκ is an oligonucleotide corresponding to an immunoglobulin κ-derived NF-κB binding site (Kieran et al., 1990). WT-PRDII is an oligonucleotide corresponding to the positive regulatory domain II (PRDII) of the CEF-4 promoter (Debbi et al., 1992). MU-PRDII is identical to WT-PRDII except for mutations in three bases that are crucial for binding of NF-κB transcription factors (Sen and Baltimore, 1986). IL-1β-κB is an oligonucleotide containing three copies of the NF-κB response element of the interleukin-1β promoter (Hiscott, 1993). The oligonucleotide GCR corresponds to the original GC-rich *cbpA* promoter sequence (see below).

Single-stranded oligonucleotides were annealed according to Sambrook et al. (1989). Two different EMSA protocols were used. In protocol A, the GCR oligonucleotide was labelled by an exchange reaction using the Klenow fragment of DNA polymerase (New England Biolabs, Beverly, MA) and [α-^{32}P]dATP (Sambrook et al., 1989), and the double-stranded oligonucleotide was purified. Gel-retardation assays were performed according to Kawata et al. (1997) with 0.1 ng of labelled probe and 2-4 μg of protein of vegetative or 16-hour nuclear extracts in the absence or presence of 2.5 ng of competitor oligonucleotide WT-PRDII, MU-PRDII or IL-1β-κB. In protocol B, Igκ and GCR oligonucleotides were end-labelled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]dATP. Free [γ-^{32}P]dATP was separated from the labelled probe using Microspin G25 microcolumns (Pharmacia PL, Uppsala, SW). Gel-retardation assays were also performed according to Kawata et al. (1997) with 1 ng of labelled probe and 3 μg of protein from 16-hour nuclear extracts in the absence or in the presence of 25 ng of competitor oligonucleotide Igκ.

Immunofluorescence

Cells at the vegetative stage or developed for 16 hours on SP-soaked nitrocellulose filters (5×10^7/ml in SP buffer, 1 ml/filter) were fixed in ice-cold methanol for 4 minutes on 12-well glass coverslips, post-fixed with 70% ethanol for 10 minutes at room temperature, and washed twice in PBS containing 0.1 M glycine. Cells were treated with 0.5% Triton X-100 in PBS for 15 minutes. Washing steps were done with PBG, i.e. PBS containing 0.5% bovine serum albumin (Boehringer, Mannheim, Germany) and 0.05% gelatin from cold water fish skin (Sigma-Aldrich, Saint Quentin Fallavier, FR), prior to incubation with primary antibodies at 4°C overnight. The purified polyclonal anti-p65 antibody from Santa Cruz was used at a concentration of 10 μg/ml. All other antibodies were used at a 1/500 dilution of the crude serum. Cells were washed in PBG and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) antibodies (Jackson Immunoresearch Lab., West Grove, PA) diluted 1000-fold for 1 hour at room temperature. DAPI (4,6-diamidino-2-phenylindol) staining was performed at 5 μg/ml for 20 minutes at room temperature. Cells were washed twice with PBG and at least four times with PBS prior to mounting the coverslips with Fluorescent Mounting Medium (Dako, Copenhagen, DK). Pictures were taken with a Leica DMRXA microscope equipped with a Leica 63× Plan Apo objective and connected to a Hamamatshu digital camera. Images were processed with Openlab 2.0.3 and Photoshop 5.0 softwares.

RESULTS

Western blot immunodetection of proteins of the NF-κB pathway

We have used a panel of antibodies specific for several components of the NF-κB pathway in mammalian cells to probe western blots of *Dictyostelium* extracts.

Denaturing western blots performed with anti-p65 antibody allowed detection of a protein with an apparent molecular mass of 58 kDa (Fig. 2, lane 1). When preincubated with saturating concentrations of its peptide immunogen (see Materials and Methods), the anti-p65 antibody was no longer able to detect any protein in cell extracts (Fig. 2, lane 7), demonstrating the

Cons.	5'-GGGPNNYYCC-3'
GCR	TTTTTTA GGGGGGCACC CCTCTTTTTTTTTCG gctttaaaaaat cccccgtgg ggagaaaaaa
Igκ	GACAGAG GGGACTTTCC GAGAGG gtctc ccctgaaagg ctctccct
IL-1β-κB	TCGACGT [GGGAAAATCC] AGGATCAGCTAGT[-]AGGATCAGCTAGTG[-]AG gca [cccttttagg] tcc[+]agtcgatca[+]tcctagtcgatcac[+]tcagct
WT-PRDII	AGCTTCT GGGAAATTCC TG aga ccctttaagg actag
MU-PRDII	AGCTTCT CTCAAATTCC TG aga gagtttaagg acctag

Fig. 1. Alignment of the DNA consensus κB binding site sequence with those of the oligonucleotides used. The κB consensus DNA binding site sequence (Cons.) (Grimm and Bauerle, 1993) is aligned with those of the oligonucleotides GCR, Igκ, IL-1β-κB, WT-PRDII and MU-PRDII (for definitions, see Materials and Methods). Bases added to the original sequences of the respective promoters are underlined. P is a purine, Y a pyrimidine, N any base. [−] in IL-1β-κB is [GGGAAAATCC] and [+] is [cccttttagg]. Complementary strand sequences are given in small letters. Bases encompassing the consensus κB binding site sequence are in bold.

specificity of the interaction (see also Fig. 3A, lane 2). When antibodies against mouse p52/p100 were used, a single protein of 100 kDa apparent molecular mass was detected (Fig. 2, lane 2). However, we failed to detect proteins reacting with anti-mammalian p50/p105 transcription factors, either with the antibody used in Fig. 2, lane 3 or with three other antibodies.

We next investigated the presence of members of the IκB family in *Dictyostelium* cell extracts. Two proteins reacting with antibodies against mouse IκBβ protein were found with apparent molecular masses of 52 kDa and 71 kDa (Fig. 2, lane 4). The 71 kDa but not the 52 kDa protein was also detected by another antibody raised against the C-terminal sequence of IκBβ (not shown). In contrast, no protein was detected in *Dictyostelium* cell extracts using anti-IκBα antibodies (not shown).

In the mammalian NF-κB pathway, IκB kinases (IKKs) are required for IκB degradation (May and Ghosh, 1998). Fig. 2 shows that *Dictyostelium* extracts contain proteins similar to the IKK family. A 81 kDa protein was detected by anti-IKKα antibodies (Fig. 2, lane 5), and a doublet (81 kDa and 79 kDa) was detected by anti-IKKβ antibodies (Fig. 2, lane 6). This doublet, also observed in some blots using mammalian extracts (Gilles Courtois, personal communication), might result from partial degradation since it was not seen in every experiment.

We also performed western blots under non-denaturing conditions, since some antibodies were raised against native proteins and the absence of a signal on denaturing western blots could be due to epitope denaturation under the conditions of electrophoresis. Fig. 3B, lane 4 indeed shows that anti-p50 antibodies revealed several strong bands under native conditions while no band was detected by the same antibody under denaturing western blot (see Fig. 2, lane 3). Although their resolution is less accurate than denaturing western blots, native western blots also permit the detection of multiprotein complexes. Using this technique with mouse cell extracts known to contain multiprotein complexes between components of the NF-κB system, several bands were detected by the anti-p65 antibody (Fig. 3A, lane 1) while only one band is detected in denaturing conditions (not shown). When preincubated with saturating concentrations of its peptide immunogen, the anti-p65 antibody was no longer able to detect any protein in cell

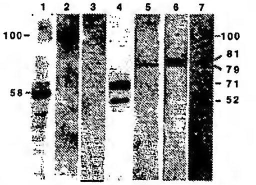

Fig. 2. Western blot detection of Rel/NF-κB-, IκB- and IKK-like proteins in *Dictyostelium* cell extracts. Cell extracts prepared as described in Materials and Methods were incubated with antibodies against mammalian p65 (lane 1) (Santa Cruz), p52/p100 (lane 2), p50/p105 (lane 3), IκB-β (lane 4), IKKα (lane 5) and IKKβ (lane 6). In lane 7, anti-p65 antibody was preincubated with an excess of its immunogen peptide prior to incubation with the membrane. The positions of molecular mass (kDa) marker proteins are shown.

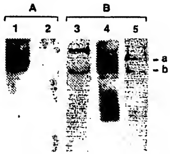

Fig. 3. Detection of *Dictyostelium* Rel/NF-κB and IκB-like proteins on non-denaturing western blots. (A) Mouse proteins revealed by anti-mammalian p65 antibody (lane 1). In lane 2, the anti-p65 antibody was preincubated with an excess of its immunogen peptide prior to incubation with the membrane. (B) *Dictyostelium* proteins revealed by antibodies against mammalian p65 (lane 3), p50 (lane 4) and IκBβ (lane 5).

extracts (Fig. 3A, lane 2), demonstrating the specificity of the interaction. When *Dictyostelium* cell extracts were submitted to native western blots (Fig. 3B), proteins located at the same positions were detected with different antibodies (Fig. 3B, lanes 3-5). For example, the bands denoted a and b in Fig. 3B reacted with at least two different antibodies indicating the presence in *Dictyostelium* extracts of multiprotein complexes comprising both p65, IκBβ and/or p50. The presence of bands that are detected with only one antibody (see Fig. 3B, lanes 3 and 4) also suggests the presence of additional yet unidentified complexes (see Discussion).

Taken together, the results from the denaturing and the native western blots strongly suggest the presence of several major components of the NF-κB system in *Dictyostelium* extracts and their association in multiprotein complexes.

Presence of protein binding to NF-κB consensus DNA sequences

We next looked by EMSA for the presence in *Dictyostelium* extracts of proteins able to bind specifically to DNA sequences similar to the NF-κB binding consensus sequences. Nuclear extracts were prepared from slug stage cells (16 hours) as described in Materials and Methods and analyzed by EMSA using different oligonucleotides. We first used an oligonucleotide (GCR) corresponding to a GC-rich region of the promoter of the *Dictyostelium cbpA* gene, coding for the developmentally regulated Ca^{2+}-binding protein CBP1 (Coukell et al., 1995). As shown in Fig. 1, this sequence displays significant homology with the κB consensus sequence 5′-GGGPuNNYYCC-3′ (Grimm and Bauerle, 1993). Fig. 4 shows the presence of a strong retarded band using labelled GCR and nuclear extracts from developing cells (lane 1).

We next examined the specificity of the protein/GCR interaction using as competitors three oligonucleotides with authentic NF-κB binding sites (WT-PRDII, IL-1β-κB and Igκ) and one oligonucleotide containing a mutated NF-κB binding sequence (MU-PRDII) (see Fig. 1). All wild-type sequences showed strong competition with the GCR probe (compare Fig. 4, lane 1 with lanes 3 and 5, and lane 6 with lane 7). In contrast, the mutant PRDII sequence did not affect appreciably the binding of the probe (compare lanes 1 and 4 in Fig. 4), again indicating the specificity of the protein/DNA interaction. Additional very minor complexes were also seen (Fig. 4, lanes 1 and 6). All of these bands were suppressed in the presence of a 25-fold excess of the wild-type competitor oligonucleotides. The occurrence of a protein(s) in *Dictyostelium* extracts binding the Igκ oligonucleotide was demonstrated further by the fact that retarded bands were also observed with 16-hour *Dictyostelium* extracts using labelled Igκ oligonucleotide (Fig. 4, lane 8). We also compared the GCR binding activity in nuclear extracts from vegetative and 16-hour-starved cells. The presence of a retarded band was seen only with nuclear extracts from developing cells (compare lanes 1 and 2 in Fig. 4), indicating a strong developmental regulation of the GCR-specific binding activity.

Immunofluorescence analysis of *Dictyostelium* NF-κB-related proteins

A characteristic property of the NF-κB transcription factors is to translocate into the nucleus upon activation of the NF-κB

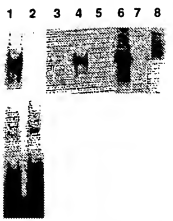

Fig. 4. Gel retardation of κB or κB-like oligonucleotides by *Dictyostelium* cell nuclear extracts. Electrophoretic mobility-shift assays performed with the ^{32}P-oligonucleotides GCR (lanes 1-7) and Igκ (lane 8) in the presence of 16-hour-starved cell nuclear extracts (lane 1, 3-8) and vegetative cell nuclear extracts (lane 2). WT-PRDII, MU-PRDII, IL-1β-κB and Igκ were used as competitors in lanes 3, 4, 5 and 7, respectively. EMSAs were performed according either to protocol A (lanes 1-5) or to protocol B (lanes 6-8), as described in Materials and Methods.

pathway. We thus compared, using immunofluorescence, the subcellular localization of homologues of two NF-κB-related transcription factors (p65 and p50) and of the IκBβ–like inhibitor in *Dictyostelium* vegetative and 16-hour developed cells. As shown in Fig. 5, while strong fluorescence was observed in all cases, different patterns were obtained depending on both the developmental stage and the antibody used. In vegetative cells, the p65-like protein was detected only in the cytosol (Fig. 5A). This labelling was specific since it decreased to background when the anti-p65 antibodies were preincubated with the peptide immunogen (Fig. 5C).

When cells starved for 16 hours were labelled with the same antibody, a completely different pattern was observed, characterized by a strong labelling of the nucleus (Fig. 5Ba). This nuclear localization of p65-like protein was further demonstrated by co-staining of the nuclei with DAPI (Fig. 5Bb). The nuclear labelling could be detected in at least 80% of the cells and was observed reproducibly in several experiments. Analogous results were obtained by performing similar experiments with anti-p50 antibodies: Fig. 5D,E shows a clear nuclear translocation of the p50-like protein in the 16-hour cells, which was also confirmed by DAPI co-staining (not shown). A completely different pattern was observed using antibodies against IκBβ. While vegetative cells were again clearly labeled in the cytosol (Fig. 5Fa), no nuclear localization in 16-hour-starved cells could be demonstrated (Fig. 5Fb,Fc), showing that nuclear translocation is restricted to transcription factor-like proteins.

The weaker cytosolic staining in developing cells compared to vegetative cells is likely to be due to the fact that developing cells were incubated with Triton X-100 after fixation to allow a better observation of the nuclear staining. Indeed, when this treatment was omitted, cells displayed a strong cytosolic staining, comparable to vegetative cells (data not shown).

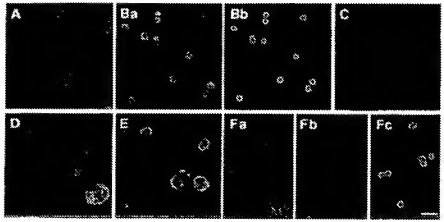

Fig. 5. Immunofluorescence detection of Rel/NF-κB and IκBβ proteins in vegetative and 16-hour-developed *Dictyostelium* cells. Vegetative cells, fixed and labelled as described in Materials and Methods, were incubated with anti-p65 antibody (A), anti-p65 antibody preincubated with an excess of its immunogen peptide (C), anti-p50 (D) and anti- IκBβ antibodies (Fa). 16-hour cells fixed and permeabilized with Triton X-100 as described in Materials and Methods were labeled with anti-p65 antibody (Ba), anti-p50 antibody (E), anti-IκBβ antibody (Fb). A DAPI staining was also performed on the cells stained with anti-p65 and anti-IκBβ antibodies (Bb and Fc). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (H+L) antibodies were used for antibody detection. Bar, 7.5 μm.

DISCUSSION

At present, only a fraction of *Dictyostelium discoideum* genome has been sequenced and current inspection of the available data bank has not revealed the presence of protein sequences highly homologous to known components of the NFκB pathway found in higher organisms. In this paper, we have used a panel of antibodies raised against mammalian components of the NF-κB pathway and oligonucleotides carrying NF-κB binding consensus sequences to investigate the presence in *Dictyostelium discoideum* cells of an homologous NF-κB pathway. We first tried to detect in *Dictyostelium* cell extracts proteins reacting with antibodies against several proteins of this pathway. The results obtained (summarized in Table 1) provide compelling evidence for the presence of elements of the NF-κB pathway in this organism. Indeed, while the presence of cross-reacting material with one antibody could always be misleading, it is striking that clear signals were obtained on western blots with several antibodies specific for different elements of the mammalian NF-κB pathway (kinases, inhibitors and transcription factors). Furthermore, most of the *Dictyostelium* proteins have molecular masses very similar to their mammalian homologues (p65, p52/p100, IKKα and IKKβ).

The anti-IκBβ antibody, however, detected 52 kDa and 71 kDa bands on denaturing western blot while only a 48 kDa protein is present in mammals (Weil et al., 1997). The 71 kDa band but not the 52 kDa band was also detected using another antibody specific for a peptide located at the carboxy-terminal end of IκBβ (not shown), strongly suggesting that this 71 kDa is the *Dictyostelium* IκBβ counterpart. The 52 kDa protein may be a cleavage product of the 71 kDa protein since the relative intensity of the two bands varied from one experiment to another.

The presence of a *Dictyostelium* IκBβ counterpart is further supported by the fact that the protein is also detected on non-denaturing western blots (Fig. 3B). The detection of bands by anti-p50 antibodies under these conditions clearly indicates that the lack of reaction on denaturing western blots is not due to the absence of the protein but to the denaturation of its epitope(s). In addition, the fact that some of the bands were detected at the same position by anti-IκBβ, anti-p50 and anti-p65 antibodies (Fig. 3B, bands a and b), indicates the presence of multiprotein complexes containing both transcription factors and inhibitor as expected for components of a NF-κB pathway. Attempts to immunoprecipitate these complexes have so far been unsuccessful, possibly because we used antibodies against mammalian proteins which may lack the high affinity required for this technique to work with *Dictyostelium* antigens.

The presence of NF-κB-like proteins in *Dictyostelium* prompted us to search for a κB-specific DNA-binding activity

Table 1. *Dictyostelium* proteins detected by cross-reaction with antibodies specific for mammalian NF-κB pathway components

Antibody anti-mammalian	Detection of proteins in *Dictyostelium* extracts		
	Denaturing WB	Native WB	kDa
p65 (RelA)	–	+	
p65* (RelA)	+	–	58
p50/p105 (NF-κB1)	–	+	
p52/p100 (NF-κB2)	+	nd	100
IκBα	–	–	
IκBβ	+	+	71, 52
IκBβ*	+	nd	71
IKKα	+	nd	81
IKKβ	+	nd	81, 79

Molecular mass (kDa) of homologues to components of the NF-κB pathway detected by the indicated antibodies raised against mammalian immunogens on native or denaturing western blots (WB).
nd, not done.
*Anti-synthetic peptide antibodies (see Materials and Methods).

in *Dictyostelium* nuclear extracts using electrophoretic mobility-shift assays (EMSAs). A κB-like oligonucleotide (GCR) was retarded by nuclear extracts from slug cells but not from vegetative cells (Fig. 4). The specificity of the interaction was demonstrated unambiguously by the fact that preincubation of the nuclear extracts with the bona-fide κB oligonucleotides Igκ, IL-1β-κB or WT-PRDII suppressed the GCR-retarded band while mutations of the guanosines required for the binding to NF-κB transcription factors on κB sequences (Sen and Baltimore, 1986) abolished the competitor activity of WT-PRDII (Fig. 4). These results strongly suggest that nuclear extracts from developing *Dictyostelium* cells contain proteins that specifically bind the κB consensus sequence. As in mammalian cells, the presence of several protein complexes is indicated by the presence of several bands both in EMSAs and in native western blots. The possibility that these bands might correspond to non-specific binding is eliminated by the fact that they were suppressed by competitor peptide or oligonucleotides.

In mammals, both p65 and p50 proteins are translocated into the nucleus upon stimulation of the Rel/NF-κB pathway and subsequent degradation of the inhibitor components of the IκB family. To further support the conclusion that the *Dictyostelium* proteins recognized by the anti-p65 and anti-p50 antibodies were slime mold homologues of NF-κB transcription factors, we investigated their subcellular localization by immunofluorescence, using the antibodies against p65 and p50 that we used in the western blot analyses. Immunofluorescence clearly indicates a nuclear translocation of the p65 and p50-related proteins that strictly parallels the presence of κB DNA binding activity in nuclear extracts, as indicated by EMSA (Fig. 5). Indeed, *Dictyostelium* p65 and p50 analogues were distributed homogeneously in the cytoplasm of all vegetative cells and their presence was never detected in the nucleus at this stage. In contrast, after 16 hours of development, both p65 and p50 were found in the cell nucleus, as confirmed by DAPI co-staining (see Fig. 5). The percentage of cells for which nuclear translocation could be demonstrated without ambiguity was somewhat variable, but it was never under 80% and was identical in prestalk and prespore cells (not shown). In contrast to p65 and p50, the IκBβ analogue was never observed to translocate into the nucleus. This nuclear exclusion of the IκBβ *Dictyostelium* counterpart is confirmed by DAPI co-staining of developing cells. It should be noted that in mammalian cells small amounts of IκBβ translocating into the nucleus were demonstrated (Suyang et al., 1996). The lack of detection of IκBβ in *Dictyostelium* cell nucleus could result either from a different behaviour of the *Dictyostelium* IκBβ inhibitor, or to the inability of the antibody used to detect faint nuclear amounts of the *Dictyostelium* protein. The punctate cytoplasmic detection of the IκBβ-like protein was similar in vegetative and late-stage *Dictyostelium* cells and western blot experiments performed on *Dictyostelium* total cell extracts showed that the IκBβ content of vegetative and 16-hour-starved cells was the same (data not shown). The reason why no disappearance of the IκBβ analogue in developing cells could be demonstrated is unclear. It is possible that the IκBβ analogue is not responsible for the cytoplasmic sequestration of p65 and p50-like proteins or, alternatively, that the cytoplasmic protein detected was readily resynthesized in 16-hour cells.

D. discoideum is a facultative metazoan which diverged very early from the main branch leading to higher eukaryotes. Nevertheless, it shares many signal transduction pathways with higher eukaryotes including the cAMP-dependent protein kinase pathway (Reymond et al., 1995) and the STAT pathway (Kawata et al., 1997). The latter, missing in *S. cerevisiae* (Hunter and Plowman, 1997), is present in *C. elegans* (Ruvkun and Hobert, 1998), indicating a possible link with multicellularity. Surprisingly, neither Rel/NF-κB nor IκB putative coding genes have been identified in *C. elegans* (Ruvkun and Hobert, 1998) and no evidence for the presence of an NF-κB pathway has been found in budding yeast (Epinat et al., 1997). In this context, the presence of *Dictyostelium* counterparts of proteins belonging to the three families constituting the mammalian NF-κB pathway, namely transcription factors, inhibitors and kinases, is quite remarkable. It should be noted that a strong homology between *Dictyostelium* NF-κB components and its mammalian counterparts can be predicted since the antibodies used in this study were selected for their ability not to crossreact with other proteins of the family and are therefore directed against non-conserved regions. This is particularly the case for anti-p65 and IκBβ antibodies, which were raised against peptides lying outside conserved RHD and ankyrin domains, respectively.

Our results provide evidence for a role of NF-κB in *Dictyostelium* development. Involvement of NF-κB in development has already been described in *Drosophila* (Miyamoto and Verma, 1995) and chicken (Bushdid et al., 1998; Kanegae et al., 1998). Although the target of the NF-κB system in *Dictyostelium* is not known, a role in the activation of the *Dictyostelium cbpA* gene is suggested by the fact that its promoter contains a κB-like site. NF-κB-like transcription factors are present in nuclei from prestalk and prespore cells at the time when CBP1 protein is expressed (Coukell et al., 1995). Interestingly, preliminary data indicate that deletion of the GCR region from the cbpA promoter leads to a loss of CBP1 expression in prespore cells, suggesting a role for this region in prespore cell differentiation (J. Pun and B. Coukell, unpublished results).

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Reconstitution of the NF-κB System in *Saccharomyces cerevisiae* for Isolation of Effectors by Phenotype Modulation

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NF-κB is a ubiquitous transcription factor that contributes to the induction of many genes playing a central role in immune and inflammatory responses. The NF-κB proteins are subject to multiple regulatory influences including post-translational modifications such as phosphorylation and proteolytic processing. A very important component of this regulation is the control of their subcellular localization: cytoplasmic retention of NF-κB is achieved through interaction with IκB molecules. In response to extracellular signals, these molecules undergo degradation, NF-κB translocates to the nucleus and activates its target genes.

To investigate novel proteins involved in this dynamic response, we have reconstituted the NF-κB/IκB system in the yeast *Saccharomyces cerevisiae*. We have successively introduced p65, the main transcriptional activator of the NF-κB family, which leads to the activation of two reporter genes controlled by κB sites, and the IκBα inhibitory protein, which abolishes this activation. By transforming such a yeast strain with a cDNA library we have performed a genetic screen for cDNAs encoding proteins capable of either dissociating the p65/IκBα complex or directly transactivating the expression of the reporter genes. The efficiency of our screen was demonstrated by the isolation of a cDNA encoding the p105 precursor of the p50 subunit of NF-κB.

We also used this system to test stimuli known to activate signalling pathways in yeast, in order to investigate whether the related mammalian cascades might be involved in NF-κB activation. We showed that yeast endogenous kinase cascades activated by pheromone, hypo- or hyperosmotic shock cannot modulate NF-κB activity in our system, and that the p38 human MAP kinase does not act directly on the p65/IκBα complex. © 1997 by John Wiley & Sons, Ltd.

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KEY WORDS — NF-κB; genetic complimentation

INTRODUCTION

The NF-κB transcription factor was identified as a DNA-binding activity specific to the decameric κB motif in the immunoglobulin κ light chain enhancer in B lymphocytes (Sen and Baltimore, 1986). Such recognition sites have been found in a great variety of genes involved in the immune and acute phase responses. The NF-κB factor is composed of homo- or heterodimers of members of the Rel family of proteins. This family is characterized by the presence of a so-called Rel homology domain of about 300 amino acids responsible for the DNA-binding activity, dimerization, nuclear localization and interaction with the IκB family of proteins (for a review, see Baeuerle and

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Henkel, 1994; Grilli *et al.*, 1993; Siebenlist *et al.*, 1994).

The Rel/NF-κB family members can be grouped into two classes: the first class includes the p105 and p100 precursor proteins, which are processed into p50 and p52 DNA-binding subunits, which represent the N-terminal part of the precursors (Bours *et al.*, 1992; Ghosh *et al.*, 1990; Kieran *et al.*, 1990; Mercurio *et al.*, 1992; Meyer *et al.*, 1991; Neri *et al.*, 1991); the other consists of the c-Rel, v-Rel, p65 and RelB proteins, as well as *Drosophila* proteins Dorsal and Dif (Ballard *et al.*, 1992; Brownell *et al.*, 1989; Ip *et al.*, 1993; Nolan *et al.*, 1991; Ruben *et al.*, 1991; Ryseck *et al.*, 1992; Stephens *et al.*, 1983; Steward, 1987; Wilhelmsen *et al.*, 1984). These classes of proteins have different abilities to activate transcription: when present as homodimers, the members of the first class (i.e. p50 and p52) are weak or inert activators; in contrast, heterodimeric complexes containing one or two subunits of the second class (c-Rel, p65, RelB) function as strong activators.

In most cell types, NF-κB is present in an inactive form in the cytoplasm via association with an inhibitory protein called IκB (for a review, see Beg and Baldwin, 1993; Blank *et al.*, 1992; Gilmore and Morin, 1993). The IκB family of proteins includes the precursor proteins p105 and p100 (Mercurio *et al.*, 1993; Morin and Gilmore, 1992; Rice *et al.*, 1992; Scheinman *et al.*, 1993), IκBα (MAD3, Haskill *et al.*, 1991), IκBβ (Thompson *et al.*, 1995), IκBγ (the C-terminal part of p105, encoded by a separate mRNA) (Inoue *et al.*, 1992a) and IκBδ (the C-terminal part of p100) (Dobrzanski *et al.*, 1995). All members of this family harbour between five and seven ankyrin-like motifs and usually interfere with nuclear localization and DNA-binding of the Rel proteins (Blank *et al.*, 1992; Nolan and Baltimore, 1992; Schmitz *et al.*, 1991). The presence of these ankyrin motifs in the C-terminus of the p105 and p100 precursors allows them to behave as IκB proteins. Each member of this class exhibits a different affinity towards the different Rel/NF-κB complexes.

Activation of NF-κB by some stimuli (PMA, TNF, IL1, LPS, hyperosmotic shock) involves phosphorylation and degradation of the IκBα and/or β molecules (for a review, see Baeuerle and Henkel, 1994; Israël, 1995; Siebenlist *et al.*, 1994; Thanos and Maniatis, 1995; G. Courtois and A. Israël, in preparation) and possibly an increased processing of the p105 and p100 precursors, which is also preceded by phosphorylation (Mellits *et al.*, 1993; Mercurio *et al.*, 1993; MacKichan *et al.*, 1996). However, the identity of the kinase(s) responsible for phosphorylation, as well as the upstream effector molecule(s) are largely unknown. We will concentrate here on IκBα, which is the most abundant and ubiquitous member of the family.

In an effort to identify some of the molecules involved in the activation pathway of NF-κB, we reconstituted part of this system in *Saccharomyces cerevisiae*. We show here that, in a yeast strain harbouring reporter genes controlled by four κB sites, we can mimic the transactivation capacity of p65 and block its activity by coexpression of IκBα. We have transformed this yeast strain with a human cDNA expression library and searched for cDNAs encoding factors able to modulate the phenotype of our test-strain, either directly through the κB sites of the reporter genes, or indirectly by preventing p65 inhibition by IκBα. This approach allowed the isolation of the p105 precursor of the p50 subunit of NF-κB, which is processed in yeast and gives rise to a p50-like protein which is poorly sensitive to IκBα and activates transcription from κB sites, therefore confirming the usefulness of our approach.

We also used this system to test several stimuli such as pheromone response, and hypo- or hyperosmotic stresses, all of which are known to activate signal transduction pathways in yeast, in order to investigate whether the related mammalian cascades might be involved in NF-κB activation. We show that the yeast endogenous kinase cascades tested cannot modulate NF-κB activity in our system and that a human protein, the p38 human MAP kinase, which is involved in the response to hyperosmotic shock in mammalian cells, does not act directly on IκBα following activation by hyperosmotic shock in yeast.

MATERIALS AND METHODS

Cells and media

All routine molecular biological techniques and culture media are described in Sambrook *et al.* (1989) and Guthrie and Fink (1991).

Yeast strain Y688κ (*MATa, leu 2-3, 112, his3-Δ200, ade2-101, URA3::4κ-lacZ, TRP1::4κ-HIS3, cyh*^{r}) is derived from Y688 (*MATa, leu 2-3, 112, trp2-901, his3-Δ200, ade1-101, can*^{r}). Y688 was transformed with a linear *Hin*dIII fragment of

pYI-4klacZ containing the *lacZ* gene downstream of four κB sites and a *CYC1* TATA box. Stable transformants were selected on medium containing 5- fluoroorotic acid and the *ura3* gene disruption verified by Southern blotting. This strain was then transformed with a linear *Eco*RV fragment of the pYκBHis plasmid (Moore *et al.*, 1993) and the stable transfonnants were selected for tryptophan prototrophy on medium lacking tryptophan. The insertion at the *trp1* locus was verified by Southern blot.

Yeast transformations

Cells were transformed using the lithium acetate method (Gietz *et al.*, 1992). For screening of the library, cells already transformed by the pS2 plasmid (see below) were retransformed with 100 μg of library DNA and double transformants were selected on ura^- leu^- trp^- his^- glucose plates. Five days after transformation, the surviving cells were tested for β-galactosidase activity using a filter lift assay: colonies were transferred onto nylon filters (Amersham N membranes), permeabilized by freezing in liquid nitrogen and thawed at room temperature. Filters were then overlaid on Whatman 3MM paper saturated with Xgal solution (1/50 solution of a 2% stock solution in dimethyl-formamide) and incubated at 30°C. The time required for color development ranged from 10 min to 2 h.

Galactose inductions

Cells from a saturated culture containing glucose as the sole carbon source were diluted at OD_{600}=0·2 in galactose minimal medium and grown to OD_{600}=1. Cells were then harvested and total protein was extracted as described below. Non-induced cultures were treated identically, but grown for 8 h in glucose minimal medium.

Stress inductions

An overnight yeast culture was diluted to OD_{600}=0·2 in glucose minimum medium and grown to OD_{600}=1·5. At this time an equal volume of either medium was added:

- the same medium supplemented with 1·8 M-NaCl or 2 M-sorbitol for hyperosmotic shock;
- the same medium supplemented with 6 μM-α-factor for stimulation of the pheromone response.

For the hypotonic stress, the culture was centrifuged and resuspended in glucose medium diluted five times in sterile water.

Aliquots were taken at the indicated times and whole cell extracts prepared as described below.

Yeast extracts

Cells were either treated as described above, or were grown in 100 ml of minimal medium to an approximate OD_{600}=1, at which time they were harvested by brief centrifugation. All following manipulations were performed at 4°C. The cell pellet was washed with water and resuspended in an equal volume of extraction buffer (20 mM-Tris pH 7·5, 1 mM-EDTA, 500 mM-NaCl, 5 mM-β-mercaptoethanol, 10% glycerol) containing protease inhibitors (PMSF at 1 mM and leupeptin, aprotinin and pepstatin at 2 μg/ml). The same volume of acid-washed glass beads was added and cells were lysed with two pulses of vortexing for 2 min separated by incubation on ice for 30 s. The cell extract was clarified by centrifugation at full speed for 15 min in a microfuge.

β-Galactosidase activity

Diluted cell extract was added to 400 μl of Z-buffer (60 mM-Na_2HPO_4, 40 mM-NaH_2PO_4, 10 mM-KCl, 1 mM-$MgSO_4$, pH 7) and 200 μl of 4 mg/ml *o*-nitro-phenyl-β-D-galactopyranoside in a final volume of 0·8 ml. Reactions were incubated at 30°C. After yellow color had developed, the reaction was stopped by the addition of 250 μl 1 M-Na_2CO_3 and the time of the reaction noted. Precipitable material was removed by brief centrifugation and the OD measured at 420 nm. The β-galactosidase units are defined as: $(2{\cdot}3 \times 10^{-7})(OD_{420}/V)(1/t)(1/m)$, where V is the volume of protein extract used in ml, t is the time of reaction in min and m is the protein concentration in mg/ml.

Western blots

Immunoblots were performed using the antisera indicated in the figure legends, followed by peroxidase-conjugated anti-rabbit immunoglobulins. For anti-haemaglutinin and anti-phosphotyrosine immunoblots, the second antibody was a peroxidase-conjugated anti-mouse immunoglobulin. Immunoreactive proteins were revealed using the Amersham ECL system.

Band shift assays

These were performed as described by Israël *et al.* (1989). DOC treatment was as described in

Bacuerle and Baltimore (1988). The following double-stranded oligonucleotides were used as probes:

Igκ: 5' tcCCTCTCGGAAAGTCCCCTCTG

GGAGAGCCTTTCAGGGGAGACag 5'

KBF: 5' gatcATGGGGAATCCCCA

TACCCCTTAGGGGTctag 5'

Plasmids

pRS316:ter/pSD06a This plasmid was a gift from S. Dalton and R. Treisman. *URA3* auxotrophy; ARS/CEN origin of replication; galactose-inducible promoter (*GAL1/CYC1*); multiple cloning site (MCS); *GAL10* terminator.

pRS315:ter *LEU2* auxotrophy; ARS/CEN origin of replication; galactose-inducible promoter (*GAL1/CYC1*); MCS; *GAL10* terminator. A *Pvu*II fragment from pSD06a containing the *GAL UAS, CYC* promoter, MCS and *GAL10* terminator sequences was cloned in pRS315 digested with *Pvu*II (Sikorski and Hieter, 1989).

pSW01 *LEU2* auxotrophy; 2 micron origin of replication; constitutive promoter (*ADH1*); MCS; *ADH1* terminator. The double-stranded oligonucleotide (containing the *Bam*HI/*Eco*RI/*Sal*I/*Hin*dIII restriction sites)

5' agctaaaattataATGGGATCCGAATTCGTCGACAAGCTT

ttttaatatTACCCTAGGCTTAAGCAGCTGTTCGAAtcga 5'

was placed in pGAD424 (Fields and Song, 1989) digested with *Hin*dIII to replace the *GAL4* activation domain with the new MCS. The sequence of the first ATG codon and the MCS are in capital letters.

pJC2 *URA3* auxotrophy; 2 micron origin of replication; constitutive promoter (*ADH1*); MCS *ADH1* terminator. A *Sph*I fragment containing the *ADH1* promoter, MCS and terminator from pSW01 was blunt-ended using DNA polymerase I Klenow fragment. This fragment was inserted into pFL44S (Bonneaud *et al.*, 1991) digested with *Hin*dIII and blunt-ended.

pRS316G:Bcl3:ter *URA3* auxotrophy; ARS/CEN origin of replication; galactose-inducible promoter (*GAL1/CYC1*); human Bcl3 cDNA; *GAL10* terminator. The Bcl3 cDNA has been amplified by polymerase chain reaction (PCR) using the following primers:

5' cggggatccCGCATGGACGAGGGGCCCGTGGAC

5' ggaattcccCTCAGCTGCCTCCTGGAGCTG

The amplification product was purified, digested with *Eco*RI and *Bam*HI and inserted into pRS316:ter digested with *Eco*RI and *Bam*HI. Sequences derived from the Bcl3 cDNA are in capital letters.

pSW01:IκBα *LEU2* auxotrophy; 2 micron origin of replication; constitutive promoter (*ADH1*); human IκBα cDNA; *ADH1* terminator. Coding sequences of IκBα were amplified by PCR using the following primers:

5' cccagatctATGTTCCAGGCGGCCGAGCGC

5' ggaattcCACTCATAACGTC

The amplification product was digested with *Bgl*II and *Eco*RI and inserted into pSW01 digested with *Bam*HI and *Eco*RI. Sequences derived from IκBα cDNA are in capital letters.

pRS316G:p65:ter *URA3* auxotrophy; ARS/CEN origin of replication; galactose-inducible promoter (*GAL1/CYC1*); human p65 cDNA; *GAL10* terminator. The coding sequence of p65 was amplified using the following primers:

5' ggggatccATGGACGAACTGTTCCCCCTC

5'gggctcgagTTAGGAGCTGATCTGACTCAG

The amplification product was digested with *Bam*HI and *Xho*I and inserted into pRS316G:ter/pSD06a digested with *Bam*HI and *Sal*I. Sequences derived from p65 cDNA are in capital letters.

pRS315G:p65:ter *LEU2* auxotrophy; ARS/CEN origin of replication; galactose-inducible promoter (*GAL1/CYC1*); human p65 cDNA; *GAL10* terminator. As for pRS316G:p65:ter, except that the amplification product was inserted into pRS315G:ter digested with *Bam*HI and *Sal*I.

pRS315G:p50:ter *LEU2* auxotrophy; ARS/CEN origin of replication; galactose-inducible promoter

(*GAL1/CYC1*); human p50 cDNA (amino acids 1–432); *GAL10* terminator. Coding sequences of p50 were amplified using the following primers:

5' gggagatctATGGCAGAAGATGATCCA

5' gggctcgagCTTCATCCCAGCATTAGATTT

The amplification product was digested with *Bgl*II and *Xho*I and inserted into pSW01 digested with *Bam*HI and *Sal*I. Sequences derived from p50 cDNA are in capital letters.

pS2 LEU2 auxotrophy; ARS/CEN origin of replication; galactose-inducible promoter (*GAL1/CYC1*); human p65 cDNA; *GAL10* terminator; constitutive promoter (*ADH1*); human IκBα cDNA; *ADH1* terminator. Sequences containing the *ADH1* promoter, human IκBα and *ADH1* terminator were excised from pSW01:IκBα with *Sph*I and inserted into pRS315G:p65:ter at the *Nae*I site upstream of the *GAL1/CYC1* promoter.

pY-p38-HA URA3 auxotrophy; 2 micron origin of replication; constitutive promoter (*ADH1*); p38-HA cDNA; *ADH1* terminator. The p38 cDNA fused to the HA epitope was amplified by PCR reaction from the pECE-p38-HA plasmid, using the following primers:

5' cccaagcttgCCGCCACCATGTAT

5' cccaagcttgggTCAGGACTCCATTTCTTCTTGGTC

The amplification product was purified, digested with *Hind*III and inserted into pJC2 at the *Hind*III site. Sequences derived from the p38-HA cDNA are in capital letters.

Detailed maps of all plasmids are available upon request.

RESULTS

Expression of Rel/NF-κB proteins

The yeast *S. cerevisiae* is a convenient host for the reconstitution of the NF-κB system, since it does not contain any endogenous NF-κB activity (Moore *et al.*, 1993; Figure 2, lane 1). To reconstitute this system we have constructed a reporter strain, Y688κ, carrying the bacterial *lacZ* and the yeast *HIS3* genes, each controlled by four κB sites cloned upstream of a minimum promoter, and integrated into the host's genome. The *lacZ* gene allows precise quantification of the activation of the promoter through the κB sites, while the *HIS3* reporter gene allows a genetic screen for the activation of this promoter, which can be monitored by using 3-aminotriazole (3-AT) as an inhibitor of growth in the absence of exogenous histidine (Durfee *et al.*, 1993; Hill *et al.*, 1986; Kishore and Shah, 1988): the higher the expression of the *HIS3* gene, the higher the concentration of 3-AT required to inhibit cell growth. We have placed p65 and IκBα cDNAs on expression plasmids under constitutive (*ADH1*) or inducible (*GAL10*) promoters respectively.

Table 1. Phenotypic analysis: liquid cultures.

Proteins	Glucose	Galactose
A		
—	0·11	0·17
IκBα (c)	0·12	0·14
p65 (i)	25	1400
IκBα (c)+p65 (i)	0·2	600
B		
p50 (i)	0·2	19
Bcl3 (i)	0·12	0·14
p50 (i)+Bcl3 (i)	0·2	20
M40 (c)	12	15

(c) indicates that the cDNA is driven by a constitutive promoter (*ADH1*) and (i) by an inducible one (*Gal10*). (A) The plasmids used to transform Y688κ are pRS316G:p65:ter for p65 (i), pSW01:IκBα for IκBα (c) and pS2 for p65 (i) plus IκBα (c). When no protein is indicated, Y688κ was transformed with pSW01 and pRS:316Gter/pSD0ßa (see Materials and Methods for details). The β-galactosidase activity was measured on total yeast crude extracts after induction in galactose or in glucose medium as indicated. Cells were collected from an overnight glucose culture, diluted to OD_{600}=0·3 in fresh glucose or galactose medium and grown to an OD_{600}=1·5. For cells expressing p65 and IκBα or IκBα alone, the induction time was 6 h; when p65 was the only protein expressed, the induction time was for a minimum of 8 h. (B) The same experiments with yeast expressing p50 and Bcl3 proteins. The plasmids used are pRS315:p50:ter for p50 (i) and pRS316G:Bcl3:ter for Bcl3 (i). The M40 cDNA is in the plasmid pAB23BX-cDNA (Schild *et al* 1990).

Phenotypic analysis indicates that p65 is recognized by the yeast transcription machinery, and that it strongly transactivates the two reporter genes (Tables 1A, 2), while it has no effect on reporter genes controlled by non-specific sites such as that for the transcription factor IRF1 (data not shown). We performed liquid cultures (Table 1) and growth tests on plates (Table 2) to validate our system: p65 acts as a strong transactivator, since it

Table 2. Phenotypic analysis: growth tests.

Proteins	Glucose		Galactose	
	+Histidine	−Histidine	+Histidine	−Histidine
−	+++	−	+++	−
IκBα (c)	+++	−	+++	−
p65 (i)	+++	+++	+++	+++
IκBα (c)+p65 (i)	+++	−	+++	++

Plasmids used were the same as in Table 1A. The growth tests were performed on plates. Yeast were collected from glucose medium plates containing histidine and streaked onto plates containing glucose or galactose as sole carbon source. Plates contained histidine as indicated. Plates were incubated at 30°C for 5 days and the ability to grow was scored as follows: '−' indicates that no colony was visible, '++' indicates the presence of a mixture of small and big colonies, '+++' indicates a normal growth rate. This test corroborates the β-galactosidase activities measured after growth in liquid culture.

allows activation even in uninduced conditions, because of the leakiness of the *GAL10* promoter. Under these conditions, coexpression of IκBα abolishes this activation (Tables 1A, 2). The use of 3-AT as growth inhibitor confirmed the results obtained by β-galactosidase measurements: cells expressing p65 driven by the *GAL10* promoter can survive a concentration of 10 mM on glucose plates lacking histidine but cannot survive a concentration exceeding 6 mM when galactose is used. This result is confirmed by the fact that liquid cultures of this strain in galactose medium do not reach stationary phase, but stop growing at $OD_{600}=2$. We conclude that p65 is toxic for yeast when it is overexpressed. If glucose is used, this strain can grow up to an OD of 10, and the untransformed strain grows as well in galactose.

The toxicity of a strong transcriptional activator (probably due to squelching of general transcription factors) has already been observed with a Gal4-VP16 hybrid molecule (Berger *et al.*, 1992). A yeast expressing p65 (*GAL10* promoter) and IκBα (*ADH1* promoter) cannot survive on glucose medium lacking histidine, but can grow on galactose medium containing less than 8 mM-3-AT. Rather paradoxically, the toxic effect of p65 results in a situation where yeast tolerates higher levels of 3-AT when less p65 is expressed: 10 mM on glucose plates (almost no expression of p65), 6 mM on galactose when only p65 is expressed (high levels of p65), and 8 mM when both p65 and IκBα are expressed. This indicates that IκBα can prevent cell death due to p65 toxicity, probably by interacting with it and by sequestering it in the cytoplasm.

The presence of the proteins as well as their interaction have been confirmed by Western blotting (Figure 1A) and gel retardation assays (Figure 2). The quantity of protein expressed is carbon-source dependent: when cells are grown under induction conditions (i.e. in medium containing galactose as the sole carbon source), p65 is detectable in Western blots (Figure 1A, lanes 6 and 8) and in gel shifts (Figure 2A, lanes 3 and 4). However, when cells are grown in glucose-containing medium, p65 is weakly or not detectable either by Western blotting (Figure 1A, lanes 5 and 7) or by retardation assays (Figure 2A, lanes 1 and 2). Despite this, the p65 activity is always detectable in growth tests or by assaying β-galactosidase activity (Tables 1A, 2). IκBα can be detected irrespective of the culture medium (Figure 1A).

The phenotypic tests show that the reporter genes are activated in a p65-dependent fashion, and that this activity is repressed when IκBα is coexpressed (Table 1A). This inhibition is indicative of IκBα-mediated p65 retention in the cytoplasm, since a mutant IκBα unable to bind p65 (*Ank4*, Inoue *et al.*, 1992b), although expressed normally, has no effect on p65-dependent transactivation (data not shown). To demonstrate that p65 is associated with IκBα as an inactive complex, we treated the extracts from cells expressing both p65 and IκBα with deoxycholate, which has been shown to dissociate the NF-κB/IκB complexes (Baeuerle and Baltimore, 1988). The increase of binding activity following detergent treatment demonstrates the presence of p65/IκBα complexes in the extract (Figure 2A, lanes 7 and 9). Under

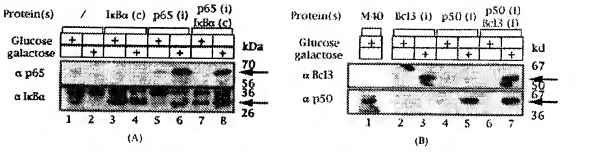

Figure 1. Western blot analysis of NF-κB/IκB proteins expressed in Y688κ. Y688κ was transformed with the indicated expression vectors. 50 μg of total extract were used in each lane. Antibodies used to reveal proteins expressed in Y688κ are: 1226 raised against human p65, 1157 raised against human p50 (Rice *et al.*, 1992), 1348 raised against the N-terminal peptide of human Bcl3 and serum S7 raised against recombinant IκBα (R. T. Hay, St Andrew, U.K.). (A) Y688κ was transformed as described in the legend to Table 1. Upper panel: p65 is detectable at a very low level when cells grow in glucose medium (lanes 5 and 7). After 8 h of induction in galactose medium, there was strong expression of the protein (lanes 6 and 8). Lower panel: the IκBα protein is detectable irrespective of the carbon source and thus the activity of the *ADH1* promoter is not modified by galactose induction. However, we can see that when yeasts are transformed with the pS2 plasmid, the IκBα level is higher in galactose than in glucose medium. This is because the *ADH1* promoter-IκBα cDNA-*ADH1* terminator cassette is cloned downstream of the *Gal10-p65-Gal10* terminator cassette on the pS2 plasmid, and this increases expression from the *ADH1* promotor when cells are grown in galactose medium. In line 6, the band with the same mobility as IκBα is non-specific and has not been found reproducibly. (B) Y688κ was transformed as described in the legend to Table 1. Like p65, p50 and Bcl3 are only detectable under inducible conditions. We also detected a strong expression of a p50-like protein from its precursor cDNA p105 isolated by the modulation of phenotype screen (M40). p105 is not processed exactly at the same position in yeast and mammals (S. Whiteside and A. Israël, unpublished data); the difference in migration with p50 in lanes 5 and 7 is due to the fact that the p50 clone used in these lanes has been arbitrarily cut at amino acid 432, which does not exactly correspond to the physiological processing site of p105; this site has not yet been localized precisely but lies around amino acid 430–435 (V. Blank and A. Israël, unpublished observation). In our experiments, only the processed p50 was detectable from the M40 clone which contains the almost complete p105 cDNA.

inducible conditions, we see a very large activation of the reporter genes by p65, but the amount of IκBα produced is not sufficient to block this activation (Table 1A).

It is important to notice that the optimal growing conditions are those in which p65 is less expressed. Under induced conditions (galactose medium), the amount of p65 produced becomes toxic. For these reasons all the modulation tests have been performed with glucose as carbon source (see below). We also tested the ability of p50 to activate gene expression in this system. As was found for p65, overexpression of p50 led to an increase in β-galactosidase activity (Table 1B). We went on to test whether the IκB protein Bcl3 (Kerr *et al.*, 1992; Naumann *et al.*, 1993; Nolan *et al.*; 1993; Ohno *et al.*, 1990) could modulate this transactivation, as has been shown to be the case in higher eukaryotes (Bours *et al.*, 1993; Fujita *et al.*, 1993; Inoue *et al.*, 1993). Unlike the results for IκBα and p65, Bcl3 was unable to modulate p50-dependent transactivation (Table 1B). This suggests that the two proteins either do not interact with each other in yeast, or that interaction does not result in transcriptional activation or inhibition.

Screening of a human cDNA library by phenotype modulation

In the previous section we have demonstrated that we can obtain a yeast strain in which p65 is complexed with IκBα, thus preventing activation of the reporter gene. In addition, we have established culture conditions whereby this yeast strain is unable to grow in medium lacking histidine, and exhibits no β-galactosidase activity. By transfecting this strain with a human cDNA library, plating on medium lacking histidine and preforming a white/blue colour assay, we have screened for factors able to modulate the NF-κB activity in our reporter strain. These factors include direct activators capable of transactivating κB sites, or indirect activators which prevent inhibition of p65 by IκBα (Figure 3).

In an initial screening of 300 000 independent clones using a human HepG2 cDNA expression library under the control of the strong and constitutive *GAP* promoter (Schild *et al.*, 1990), 124 putative positives (His^+ and blue) were isolated. These clones showed a range of blue colour, indicating that several classes of modulating factors may be present. Three cDNAs which encode p105,

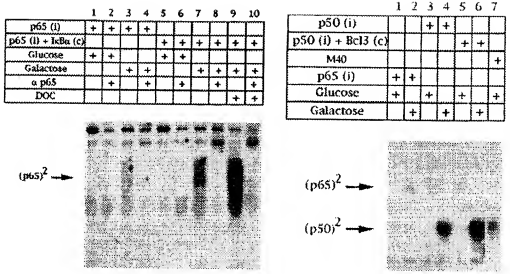

Figure 2. Analysis of NF-κB binding activity in Y688κ. Band shift assay using whole cell extracts from transformed yeast strains. Y688κ was transformed as described in the legend to Table 1. α-p65 indicates that the 1226 antibody raised against p65 has been added to the binding reaction. DOC indicates that 0·2% DOC has been included in the binding reaction following NP40 (Baeuerle and Baltimore, 1988). 20 μg of total extract was used in each lane. (A) The electrophoretic mobility shift assay was performed with the KBF probe. Specific binding of p65 can be detected under induced conditions (compare lanes 1 and 3). The 1226 serum blocks the appearance of the p65/p65 complex (lanes 4, 8 and 10). When IκBα is coexpressed, it forms complexes with p65, which are released by deoxycholate treatment (compare lanes 7 and 9). Western blots show that the difference in binding observed between lanes 3 and 9 is due to a lower expression level of p65 in the cells expressing p65 alone than in the cells expressing p65 and IκBα, after galactose induction in this experiment (data not shown and see text). (B) Gel shift experiments using the Igκ probe show that p50 binding is detectable under induced conditions (lanes 4 and 6) and that Bcl3 coexpression does not modify its binding. The p50-like protein derived from the p105 precursor alone isolated from the HepG2 cDNA library also binds to the Igκ site (M40, lane 7).

the p50 precursor, were isolated. One of them, M40, corresponds to a 2·8 kb cDNA encoding a partial p105 molecule lacking a small portion of the C-terminal region. Western blot and gel shift assays showed that p105 is processed to a p50-like form in yeast cells and that the resulting p50 can form homodimers which are able to bind DNA and transactivate the reporter genes (Table 1B, Figures 1B, lane 1, and 2B, lane 7). No precursor p105 protein is detectable, suggesting that the truncated p105 molecule encoded by M40 is entirely processed in yeast. We are currently analysing other cDNAs isolated during this screen.

Effect of activating yeast signal transduction pathways on the reconstituted system

In higher eukaryotes, NF-κB activity is induced by a great variety of extra-cellular stimuli. These stimuli activate kinase cascades leading to the phosphorylation and subsequent degradation of IκBα. It has been demonstrated that many kinase cascades are conserved between mammals and yeast (Errede and Levin, 1993; Marshall, 1994; Neiman, 1993). In an attempt to identify activators of the NF-κB system, we treated our yeast test strain with various stimuli known to activate endogenous yeast pathways (for a review, see Herskowitz, 1995), and assayed whether this resulted in an increased NF-κB-dependent β-galactosidase activity.

Three distinct protein kinase cascades activate members of the MAPK group in yeast (Herskowitz, 1995): the pheromone response pathway, the cascade that controls response to high osmolarity and the cell wall integrity module. Firstly, we tested the pheromone-induced response

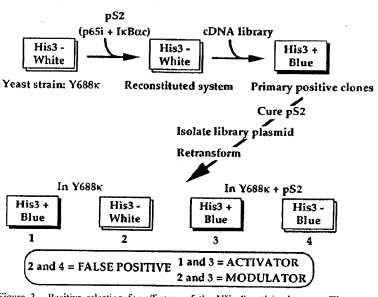

Figure 3. Positive selection for effectors of the NF-κB activity in yeast. The yeast strain containing two reporter genes under the control of four κB sites is transformed with the pS2 vector. The phenotype of this transformed strain is the same as an untransformed Y688κ. A cDNA expression library is then transformed and positive clones are selected if they show the phenotype of a strain that contains p65 only. Additional genetic tests allow the discrimination of the primary positive clones. Phenotypes are indicated in rectangles. p65i and IκBαc indicate that p65 and IκBα are under the control of an inducible and a constitutive promoter, respectively.

by incubating the yeast culture with the mating α-factor (3 μM final concentration) to activate the FUS3/KSS1 MAP kinase homologue. This pathway shows some similarities with the SRF-linked signalling pathway (Hill *et al.*, 1995). We also tested hypotonic stress, which can activate the MPK1 kinase downstream of the PKC1 pathway (Paravicini *et al.*, 1992; Kamada *et al.*, 1995) by transferring a log phase culture into 20% minimum medium solution. Neither of these stimuli produced an activated phenotype (induction of β-galactosidase activity) nor modification of IκBα quantity nor mobility in Western blotting analysis (data not shown).

Preliminary studies in our laboratory have shown that a hyperosmotic stress can induce NF-κB activity in mammalian cells, via IκBα degradation (G. Courtois and A. Israël, in preparation). Hyperosmotic shock activates the p38 MAP kinase in mammalian cells, although there is no evidence that this kinase is directly implicated in NF-κB activation. The homologue of p38 in yeast is HOG1, and p38 can complement a *hog1* deletion mutant (Han *et al.*, 1994). We therefore activated the HOG1 pathway (Brewster *et al.*, 1993) in our test strain with a hyperosmotic stress (0·9 M-NaCl or 1 M-sorbitol for 0, 5, 15, 30, 45, 60 and 120 min) and assayed the effect upon p65-dependent transactivation and levels of IκBα protein. The activation of the cascade was monitored by anti-phosphotyrosine Western blotting to detect the appearance of activated, phosphorylated HOG1. We were unable to see any phenotype modulation or any variation in the level or mobility of IκBα (which would be indicative of phosphorylation).

One possibility to explain this negative result is that due to species differences, HOG1 cannot recognize the IκBα protein. We therefore tried to cotransform Y688κ with pS2 and a plasmid carrying the p38 cDNA (tagged with the influenza haemaglutinin epitope) under the control of the *ADH1* promoter. We stimulated this strain with a hyperosmotic shock for 5, 15, 30 and 60 min and investigated whether we could activate p38 kinase and thus induce IκBα phosphorylation or degradation. With these two plasmids, in glucose medium, the level of each protein (p65, IκBα and

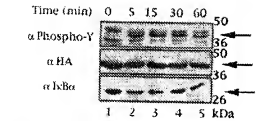

Figure 4. Western blot analysis of p38 following hyperosmotic shock on a yeast strain expressing IκBα. Y688κ was transformed with pSW01:IκBα and pY-p38-HA. 50 μg of total extract were used in each lane. An overnight culture was diluted to OD_{600} ±0·2 in glucose minimum medium and grown to OD_{600}=1·5. At this time an equal volume of the same medium supplemented with 1·8 M-NaCl was added. Aliquots were taken at the indicated times and total protein extracts prepared as described. The level of the p38-HA protein, followed using the 12CA5 mAb raised against the HA tag, remained identical throughout the time course (middle panel). After 5 min of induction the level of tyrosine phosphorylated p38, monitored with the 4G10 mAb raised against phosphotyrosine, was maximal and decreased after 45 min (upper panel). No effect was detectable either on the expression level of the IκBα protein or on its mobility, indicating that its phosphorylation state or its stability were not modified by induction of p38 activity (lower panel). The upper band of the lower panel is non-specific and is also seen with an extract derived from untransformed cells (data not shown).

p38-HA) was very low, probably because of an excess of heterologous expression. However, the β-galactosidase activity remained identical during the experiments, indicating that the amount of free p65 did not change (data not shown).

We decided to use Y688κ strain transformed with pSW01:IκBα and pY-p38-HA as a tool to investigate the levels and mobility of IκBα by Western blot during the hyperosmotic shock. While p38 hyperphosphorylation was readily detectable following hypertonic stress (Figure 4, upper panel), indicating that the kinase was activated, no effect on the IκBα molecule could be observed (Figure 4, lower panel). In addition, experiments in which JNKK (MKK4) was coexpressed in yeast cells with IκBα were performed. This molecule can partially complement a *pbs2*$^{-}$ yeast strain (Lin *et al.*, 1995). The *PBS2* gene, like the *HOG1* gene, is necessary for cells to grow at high osmolarity. It encodes a MEK homologue (MAPKK) which rapidly activates the HOG1 protein (MAPK homologue) by tyrosine phosphorylation when cells are exposed to external osmolarity (Brewster *et al.*, 1993). In mammalian cells, JNKK functions as a MAPKK and activates the Jun kinases and the p38 MAPK (Derijard *et al.*, 1995). We would like to see if this MAPKK could modulate our system when activated, whether directly by modifying IκBα or indirectly by overactivating the HOG endogenous pathway. No change in IκBα mobility was evident following hyperosmotic shock (data not shown). These experiments show that the yeast endogenous kinase pathways cannot modulate NF-κB activity in our system and that p38 kinase does not directly phosphorylate IκBα.

DISCUSSION

We describe here the reconstitution in yeast of the mammalian NF-κB/IκBα system. The transactivating factor p65 can activate two distinct reporter genes and its activity can be blocked by the coexpression of its specific inhibitor, IκBα. According to the proteins expressed, we were able to define two distinct phenotypes: an activated phenotype and an inhibited phenotype. The activated phenotype can be observed in the following cases: when the p65 is expressed alone, in either galactose- or glucose-containing medium (the latter results from the leakiness of the *GAL10* promoter in the presence of glucose), and when p65 is overexpressed in combination with IκBα. The inhibited phenotype is seen when p65 and IκBα are coexpressed in glucose medium (so that IκBα is expressed more strongly than p65). In this latter case, the total amount of p65 is complexed with IκBα, as judged by the activation of DNA-binding activity following treatment of a cell extract with deoxycholate (not shown). The p65 thus sequestered is therefore unable to activate transcription. These conditions reconstitute the latent state of the NF-κB/IκB proteins in a non-stimulated mammalian cell.

By transforming our reporter strain with a human cDNA library cloned in a yeast expression vector and screening for the activated phenotype, we searched for potential new members of the activation pathway able to induce the nuclear translocation of NF-κB. This approach also permits the isolation of clones encoding direct transactivators of the reporter genes. As we needed to screen the library on non-inducible medium, we chose one where cDNAs were driven by the strong constitutive *GAP* promoter. Cells were transformed and 300 000 independent clones obtained on selective glucose medium lacking histidine. Our preliminary results indicate that three clones from our primary screening encode the p105 precursor protein. This confirms that this system allows the

isolation of direct transactivators. The other type of modulators can only be isolated assuming that they alone can act on the p65/IκBα complex and that they are constitutively active in yeast cells. Despite these reservations, our reconstituted system allows a very simple and rapid test of any molecule thought to be involved in the last step of NF-κB activation.

We also attempted to reconstitute the interaction of p50 with the IκB-like molecule Bcl3, which results either in inhibition of binding or in formation of a ternary complex with DNA, according to different authors (Bours *et al.*, 1993; Fujita *et al.*, 1993; Inoue *et al.*, 1993). Since both cDNAs were placed under the control of the *GAL10* promoter, the two proteins are only detectable when galactose is used as the sole carbon source. As in mammalian cells, p50 homodimers are weak transactivators. However, the coexpression of the Bcl3 protein did not modify p50 DNA-binding activity nor transactivation, indicating that either these proteins are not able to interact with each other in yeast, or that this interaction does not result in a modification of transcriptional activation or DNA-binding activity. It has been shown that the activity of Bcl3 in mammalian cells is strongly dependent on phosphorylation (Fujita *et al.*, 1993). This modification may not take place correctly in yeast, therefore precluding biological activity. Bcl3 protein can, however, transactivate a *LacZ* reporter gene when bound to DNA via a Gal4 DNA-binding domain, suggesting that it is the interaction between p50 and Bcl3 which is somehow impaired in the yeast system (data not shown).

Another way to investigate the activation pathway that allows the dissociation of the p65/IκBα complex is to activate *S. cerevisiae* by stimuli known to participate in transduction cascades. We used three different stimuli which activate yeast kinase cascades: pheromone activation using α-factor, hypotonic stress and hyperosmotic stress. None of these three signals resulted in transcriptional activation in our system. One possibility is that none of the pathways we activated is physiologically relevant for NF-κB activation. However, work in our laboratory (G. Courtois and A. Israël, in preparation) indicates that hyperosmotic shock in mammalian cells is able to induce NF-κB nuclear translocation following phosphorylation and degradation of IκBα, and it has been shown that the cascade induced by hyperosmotic shock is conserved between yeast and mammals (Galcheva-Gargova *et al.*, 1994; Ham *et al.*, 1994; Maeda *et al.*, 1995). Another possibility is that the last protein of the cascade (most likely a kinase) does not recognize the IκBα molecule, due to species differences. To circumvent this particular problem we used the hyperosmotic shock but in addition cotransformed our yeast strain with a plasmid carrying the mammalian p38 MAP kinase cDNA. This molecule is homologous to the yeast HOG1 kinase, which is activated by hyperosmotic shock, and can complement a *hog1* deletion mutant, indicating that the signal transduction pathway in yeast following hyperosmotic stress acts in a manner analogous to the mammalian pathway. Under these conditions we were able to activate the phosphorylation of the p38 protein which was maximal after 5 min and decreased after 45 min. During this time, the level of expression of p38 remained the same. When we examined IκBα by Western blotting, we could detect no modification either in the total amount nor in the mobility of the protein (which would be indicative of a modification of its phosphorylation state). We therefore conclude that the p38 MAP kinase is most likely not involved in the activation of NF-κB in mammalian cells during a hyperosmotic stress. Alternatively, it may be that an additional protein is required, which is not present or active in yeast during our experiments. As mentioned previously, our reconstituted system provides an easy assay for testing stimuli or specific proteins that are postulated to be involved in NF-κB signalling.

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Immunology

NF-κB-inducing kinase activates IKK-α by phosphorylation of Ser-176

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ABSTRACT **Activation of the transcription factor NF-κB by inflammatory cytokines involves the successive action of NF-κB-inducing kinase (NIK) and two IκB kinases, IKK-α and IKK-β. Here we show that NIK preferentially phosphorylates IKK-α over IKK-β, leading to the activation of IKK-α kinase activity. This phosphorylation of IKK-α occurs specifically on Ser-176 in the activation loop between kinase subdomains VII and VIII. A mutant form of IKK-α containing alanine at residue 176 cannot be phosphorylated or activated by NIK and acts as a dominant negative inhibitor of interleukin 1- and tumor necrosis factor-induced NF-κB activation. Conversely, a mutant form of IKK-α containing glutamic acid at residue 176 is constitutively active. Thus, the phosphorylation of IKK-α on Ser-176 by NIK may be required for cytokine-mediated NF-κB activation.**

Many of the common proinflammatory properties of tumor necrosis factor (TNF) and interleukin 1 (IL-1) are mediated by the transcription factor NF-κB (1–3). Under normal conditions, NF-κB exists in a cytoplasmic complex with an inhibitor protein IκB (1–3). The activation of NF-κB requires phosphorylation of IκB-α at Ser-32 and Ser-36 (4). This phosphorylation targets IκB-α for ubiquitination and proteosome-mediated degradation, thereby releasing NF-κB to enter the nucleus and activate a series of genes involved in the inflammatory response (5).

It is now known that NF-κB activation by TNF and IL-1 involves signal transduction cascades containing several intermediate signaling proteins. TNF initiates its signaling by binding to and trimerizing the type 1 TNF receptor, TNF-R1 (6, 7). Several cytoplasmic proteins, including TNF-R1-associated death domain protein (TRADD) (6), TNF receptor-associated factor (TRAF2) (8), and receptor-interacting protein (RIP) (9), are then recruited to the intracellular domain of TNF-R1 where they form an active signaling complex. Overexpression of each of these proteins can activate the signaling cascade leading to NF-κB activation. On the other hand, IL-1 induces the formation of a complex including two distinct receptor chains, IL-1R1 and IL-1RAcP (10), the adaptor protein MyD88 (11), and the protein kinase IRAK (12). Following its activation, IL-1 receptor-associated kinase (IRAK) is released from the receptor complex (11) and associates with TRAF6 (13).

These distinct TNF and IL-1 pathways merge at the level of the protein kinase NF-κB-inducing kinase (NIK) (14). NIK, which is a member of the mitogen-activating protein (MAP) kinase kinase kinase (MAP3K) family, was originally identified as a TRAF2-interacting protein. NIK activates NF-κB when overexpressed, and kinase-inactive mutants of NIK behave as dominant-negative inhibitors that suppress NF-κB activation mediated by TNF, IL-1, TRADD, RIP, TRAF2, TRAF5, and TRAF6 (14, 15). Thus, NIK is a common mediator in the NF-κB signaling cascades triggered by TNF and IL-1 that acts downstream of the receptor complexes. However, NIK is not involved in TNF and IL-1-stimulated kinase pathways that lead to the activation of the Jun N-terminal kinase (15). The details of the molecular mechanism(s) by which NIK itself becomes activated are not yet understood.

In an effort to identify downstream targets of NIK, conserved helix–loop–helix ubiquitous kinase (CHUK) was isolated in a yeast two-hybrid screen (16). CHUK was also biochemically purified from TNF-treated HeLa cells by DiDonato *et al.* (17) and by Mercurio *et al.* (18). CHUK is an 85-kDa protein kinase of previously unknown function (19). CHUK directly associates with, and specifically phosphorylates IκB-α on Ser-32 and -36 (16, 17). A catalytically inactive mutant of CHUK is a dominant-negative inhibitor of TNF-, IL-1, TRAF2-, TRAF6-, and NIK-induced NF-κB activation and CHUK kinase activity is stimulated by cytokine treatment (16, 17). Based on these results, CHUK has been redesignated as IκB kinase-α (IKK-α). Cells cotransfected with NIK and IKK-α display elevated IKK-α activity (16). A second IKK, IKK-β, with 52% amino acid identity to IKK-α, was also recently identified (18, 20, 21). IKK-α and IKK-β exist in a heterocomplex form that is able to interact with NIK (20). Thus, IKK-α and IKK-β may both represent downstream targets of NIK, and all three of these kinases are likely present in a large IκB kinase complex of 700–900 kDa (17, 20, 22).

Because NF-κB can be activated by numerous stimuli, the IKK complex may serve as an integration point for signals emanating from many different pathways. Although the molecular mechanism for activation of this kinase complex is not known, IKK-α is likely a downstream target of NIK, because NIK coexpression stimulates the ability of IKK-α to phosphorylate IκB-α. Additionally, a dominant negative form of IKK-α blocks NIK-induced NF-κB activation (16, 20). Because NIK is a member of the MAP3K family, it may activate downstream kinases by specific phosphorylation events similar to other MAP3K family members. In this study, we report that IKK-α is a better substrate than IKK-β for phosphorylation by NIK. The primary site of IKK-α phosphorylation by NIK is Ser-176 in the kinase activation loop. Phosphorylation of this residue correlates with activation of IKK-α.

MATERIALS AND METHODS

Cell Culture and Biological Reagents. Recombinant human TNF and IL-1 were provided by Genentech, Inc. (South San Francisco). The anti-FLAG mAb M2 affinity resin and purified FLAG peptide were purchased from Eastman Kodak. Rabbit anti-FLAG and anti-Myc polyclonal antibodies were from Santa Cruz Biotechnology. Human embryonic kidney

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Abbreviations: NIK, NF-κB-inducing kinase; IKK, IκB kinase, TNF, tumor necrosis factor; IL, interleukin; MAP, mitogen-activating protein; MAP3K, MAP kinase kinase kinase; β-gal, β-galactosidase.

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293 cells, 293/IL-1R1 cells (12) and HeLa cells were maintained as described (6).

Expression Vectors. Mammalian cell expression vectors encoding wild-type and kinase-inactive versions of NIK, IKK-α, and IKK-β have been described (15, 16, 20). IKK-α(KA) and IKK-β(KA) signify lysine to alanine changes at amino acid 44 of IKK-α and IKK-β, respectively. NIK(KA) represents lysine to alanine changes at amino acids 429 and 430 of NIK. The control expression plasmid pRK5, the NF-κB-dependent E-selectin-luciferase reporter gene plasmid and plasmid RSV-β-galactosidase were also described (23, 24). Expression vectors encoding IKK-α and IKK-β mutants with alanine or glutamic acid replacing serine and threonine residues in the activation loop of IKK-α and IKK-β [IKK-α(S176A), IKK-α(KA)S176A, IKK-α(S176E), IKK-α(T179A), IKK-α(KA)T179A, IKK-α(S180A), IKK-α(KA)S180A, IKK-β(S177A)] were constructed using Stratagene Quickchange site-directed mutagenesis kit. All the mutations were verified by DNA sequencing analysis.

Immunoprecipitation, Western Blot Analysis and *in Vitro* Kinase Assays. 293 cells or HeLa cells were transiently transfected with expression plasmids by using calcium phosphate as described (6). Between 24–36 hr later, cells were washed with cold PBS and lysed in Nonidet P-40 lysis buffer containing 50 mM Hepes (pH 7.6), 250 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% Nonidet P-40, and Complete protease inhibitors (Boehringer Mannheim) (16, 20). Cell lysates were cleared and incubated for 2–4 hr at 4°C with anti-FLAG M2 antibody resin (Kodak), washed extensively with lysis buffer and eluted with FLAG peptide (300 μg/ml, Kodak) or not eluted. *In vitro* kinase assays were performed with eluted proteins or immune complexes and bacterially synthesized IκB-α (amino acids 1–250) proteins (16) in 20 μl kinase buffer containing 20 mM Tris·HCl (pH7.6), 10 mM MgCl2, 0.5 mM DTT, 100 μM ATP, and 5 μCi of [γ-^{32}P]ATP (1Ci = 37 GBq) at room temperature for 30 min (16). Samples were analyzed by 10% SDS/PAGE and autoradiography. Immunoblotting analyses were performed with rabbit polyclonal antibodies and detected by alkaline phosphatase-conjugated goat-anti-rabbit secondary antibody.

Reporter Assays. For reporter gene assays, 293 cells, 293/IL-1R1 cells, or HeLa cells were seeded into six-well plates. Cells were transfected the following day by the calcium phosphate precipitation method with 0.5 μg E-selectin-luciferase reporter gene plasmid, 1 μg pRSV-β-gal plasmid, and various amounts of each expression construct. The total DNA transfected (4.5 μg) was kept constant by supplementation with the control vector pRK5. In the NIK and IKK-α synergy experiments, 0.01 μg of NIK, IKK-α(WT), and IKK-α(S176A) were used for each 35-mm well. After 24 hr, cells were either left untreated or stimulated with IL-1 (10 ng/ml), or TNF (100 ng/ml) for 5 hr prior to harvest. Reporter gene activity was determined with the Luciferase Assay System (Promega). The results were normalized for transfection efficiency on the basis of β-gal expression.

RESULTS

***In Vitro* Phosphorylation of IKK-α by NIK.** NIK directly interacts with IKK-α and IKK-β, and the phosphorylation of IκB-α by IKK-α and IKK-β is enhanced by NIK coexpression (16, 20). These results suggest that IKK-α and IKK-β may be NIK-activated IκB-α kinases that link TNF-and IL-1-induced kinase cascades to NF-κB activation. To investigate if IKK-α and IKK-β can be phosphorylated by NIK, we transiently expressed FLAG epitope-tagged wild-type or kinase-inactive mutants of NIK, IKK-α, and IKK-β in human embryonic kidney 293 cells (Fig. 1). The epitope-tagged proteins were immunoprecipitated with an anti-FLAG antibody, and incubated with [γ-^{32}P]ATP. In these assays, wild-type IKK-α, IKK-β, and NIK become autophosphorylated when expressed individually, while mutants of all three kinases containing lysine-to-alanine (KA) substitutions in their ATP-binding sites were not autophosphorylated (16). The IKK-α(KA) and IKK-

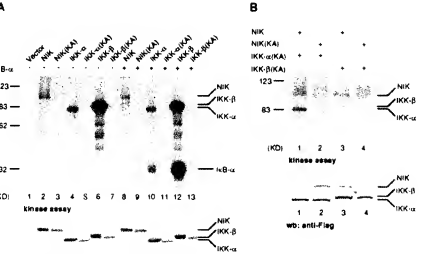

FIG. 1. In vitro phosphorylation of IKK-α by NIK. (*A*) Autophosphorylation and phosphorylation of IκB-α by various kinases. 293 cells were transiently transfected with expression plasmids encoding FLAG epitope-tagged wild-type or KA mutants of NIK, IKK-α and IKK-β. Thirty-six hours after transfection, extracts were immunoprecipitated with anti-FLAG mAb affinity resin and FLAG-tagged proteins were purified as described in *Materials and Methods*. Purified proteins were incubated with [γ-^{32}P]ATP in the presence or absence or bacterially synthesized protein IκB-α (amino acids 1–250), resolved by SDS/PAGE, and analyzed by autoradiography. The amounts of proteins used in the reactions were determined by immunoblotting (wb) with anti-FLAG polyclonal antibodies (*Lower*). The positions of IKK-α, IKK-β, and NIK are indicated. (*B*) Phosphorylation of IKK-α(KA) and IKK-β(KA) by NIK. 293 cells were transiently transfected with expression plasmids encoding FLAG epitope-tagged wild-type NIK, IKK-α(KA), or IKK-β(KA). Purified proteins were incubated with [γ-^{32}P]ATP, resolved by SDS/PAGE, and analyzed by autoradiography. The amounts of proteins used in the reactions were determined by immunoblotting (wb) with anti-FLAG polyclonal antibodies (*Lower*). The positions of IKK-α, IKK-β, and NIK are indicated.

β(KA) mutants were also unable to phosphorylate IκB-α (Fig. 1*A*).

To examine its ability to phosphorylate IKK-α and IKK-β, we coexpressed NIK with the catalytically inactive IKK mutants. We found that NIK can phosphorylate IKK-α(KA), but only weakly phosphorylates IKK-β(KA) (Fig. 1*B*, compare lanes 1 and 3). The kinase-inactive NIK does not phosphorylate IKK-α(KA) or IKK-β(KA) (Fig. 1*B*, lanes 2 and 4). In addition, we found that purified, baculovirus-expressed IKK-α(KA) can be phosphorylated by baculovirus-expressed NIK, but not by baculovirus-expressed NIK(KA) (data not shown).

Ser-176 of IKK-α Is Phosphorylated by NIK. Because NIK is a MAP3K-related kinase, it may activate a downstream kinase or kinases in a manner similar to other members of the MAP3K family. MAP3Ks activate MAP2Ks (such as MEK1) by phosphorylating serine and threonine residues in the "activation loop" between kinase subdomains VII and VIII (25–27). Therefore, we examined serine and threonine residues in the activation loop of IKK-α as we have no evidence for IKK-α being tyrosine-phosphorylated (unpublished data).

There are two serines (residues 176 and 180) and a threonine (residue 179) in the activation loop of IKK-α (Fig. 2). To test if Ser-176, Thr-179, or Ser-180 are phosphorylated by NIK, each of these three residues was mutated to alanine in the background of the inactive IKK-α(KA) mutant to eliminate IKK-α autophosphorylation activity, and tested for phosphorylation by NIK. Mutation of Ser-176 to alanine [IKK-α(S176A)] significantly reduced the phosphorylation of IKK-α by NIK, while the T179A and S180A mutants were still efficiently phosphorylated (Fig. 3). These results indicate that Ser-176 represents the major site of IKK-α phosphorylation by NIK.

Loss of Activation of IKK-α(S176A) by NIK. Because NIK can phosphorylate Ser-176 in the activation loop of IKK-α and stimulate IκB-α phosphorylation by IKK-α, it is possible that Ser-176 phosphorylation may be required for IKK-α activity. If so, the phosphorylation of IκB-α by IKK-α should be greatly impaired when Ser-176 of wild-type IKK-α is mutated to alanine. IKK-α(S176A) was expressed, purified, and found to have greatly reduced activity as measured by both its autophosphorylation and its ability to phosphorylate IκB-α (Fig. 4*A*). In contrast, mutation of the equivalent serine in IKK-β results in a kinase, IKK-β(S177A), that is fully active in autophosphorylation and in phosphorylation of IκB-α (Fig. 4*B*).

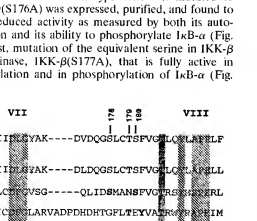

FIG. 2. Alignment of IKK-α amino acid sequences with several kinases in the activation loop region. The D(F/L)G and (A/S)PE residues that are characteristic of kinase subdomains VII and VIII are shaded. The conserved threonine and tyrosine residues in the TXXY motif adjacent to subdomain VIII are also shaded. The activating phosphorylation sites in MEK1 (25, 26), MAPK (30), PKA (31), CDK2 (32), and PKC-α (33) are shown in boldface. The position of the serine and threonines residues of IKK-α are indicated. The sequence of the activation loop of IKK-β is also included.

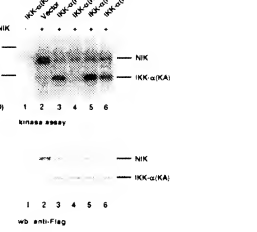

FIG. 3. Ser-176 in the activation loop of IKK-α is a major site of phosphorylation by NIK. Individual serine and threonine residues in the activation loop of IKK-α kinase domain were mutated to alanine. Each IKK-α mutant protein also contained the KA mutation in the ATP-binding site to prevent autophosphorylation. 293 cells were transiently transfected with expression plasmids encoding the indicated FLAG epitope-tagged proteins. Thirty-six hours after transfection, immunoprurified proteins were incubated with [γ-^{32}P]ATP, resolved by SDS/PAGE, and analyzed by autoradiography. The amount of protein used in each reaction was determined by immunoblotting (wb) with anti-FLAG polyclonal antibodies (*Lower*).

To determine whether IKK-α(S176A) is also defective in NF-κB activation, we compared IKK-α and IKK-α(S176A) in an NF-κB reporter gene assay in transiently transfected HeLa cells. As expected (16), expression of IKK-α modestly activated the NF-κB luciferase reporter gene in a dose-dependent manner. Mutation of Ser-176 to alanine abrogated the ability of IKK-α to activate NF-κB, similar to the IKK-α(KA) mutant (Fig. 4*C*).

If phosphorylation of Ser-176 is required to activate IKK-α, then mutation of this site should impair the ability of IKK-α to be activated by NIK. To test this, we coexpressed either FLAG epitope-tagged IKK-α or IKK-α(S176A) with Myc epitope-tagged wild-type NIK. We then specifically immunopurified the FLAG epitope-tagged IKK-α proteins and assayed them for IκB-α phosphorylation activity in an *in vitro* kinase assay. The phosphorylation of IκB-α by IKK-α was significantly enhanced when IKK-α was stimulated by NIK, but NIK failed to activate IKK-α(S176A) kinase activity to a similar extent (Fig. 5*A*).

The inability of NIK to activate IKK-α(S176A) is not only reflected by *in vitro* kinase assay but is also observed in tissue culture cells by using an NF-κB reporter gene assay. As shown in Fig. 5*B*, low levels of NIK and IKK-α synergistically activated the NF-κB luciferase reporter gene when coexpressed. This synergy was not observed when NIK is coexpressed with IKK-α(S176A).

IKK-α(S176A) Is a Dominant Negative Inhibitor of IL-1- and TNF-Induced NF-κB Activation. IKK-α associates with both NIK and IKK-β (16, 20). Because the IKK-α(S176A) mutant is inactive in both kinase and NF-κB reporter assays, it might compete with endogenous IKK-α for binding to NIK, IKK-β, or IκB-α and thereby block the activation of NF-κB. To test this, we determined the effect of IKK-α(S176A) on IL-1- and TNF-induced NF-κB activation in reporter gene assays in 293/IL-1R1 cells. As shown in Fig. 6, overexpression of IKK-α(S176A) blocked both IL-1- and TNF-induced reporter gene activation in a dose-dependent manner. In addition, overexpression of IKK-α(S176A) blocked NIK-, TRAF2-, and

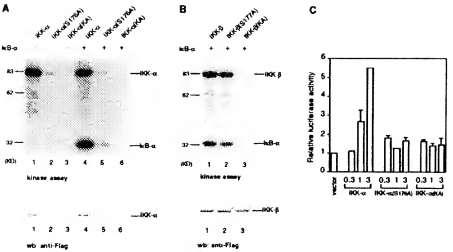

FIG. 4. IKK-α(S176A) has reduced kinase activity and NF-κB activation. (*A*) IKK-α(S176A) has reduced kinase activity. 293 cells were transiently transfected with the indicated epitope-tagged expression vectors. Thirty-six hours after transfection, IKK-α proteins were immunopurified with anti-FLAG mAb affinity resin and used in *in vitro* kinase reactions with IκB-α and [γ-^{32}P]ATP. (*Lower*) The protein expression in each lane is shown. (*B*) IKK-β(S177A) has similar kinase activity as IKK-β. 293 cells were transiently transfected with the indicated epitope-tagged expression vectors. Thirty-six hours after transfection, IKK-β proteins were immunopurified with anti-FLAG mAb affinity resin and used in *in vitro* kinase reactions with IκB-α and [γ-^{32}P]ATP. (*Lower*) The protein expression in each lane is shown. (*C*) IKK-α(S176A) is defective in NF-κB activation. HeLa cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α expression vector as indicated. Twenty-four hours after transfection, luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages (±SEM) of duplicate samples for one representative experiment.

TRAF6-induced reporter gene activation in a dose-dependent manner (data not shown).

Mutation of Ser-176 to Glutamic Acid Activates IKK-α. Phosphorylation of IKK-α at Ser-176 introduces negative charge into this portion of the protein and results in kinase activation. It has been shown that substitution with negatively charged amino acids in the activation loop of other kinases can mimic activation (26, 27). Therefore we constructed an IKK-α(S176E) mutant, which contains glutamic acid at position 176. We expressed different doses of IKK-α or IKK-α(S176E) in HeLa cells, and measured the ability of immunopurified IKK-α or IKK-α(S176E) to phosphorylate IκB-α. At equivalent levels of expression, IKK-α(S176E) had significantly greater kinase activity than IKK-α, as measured by either autophosphorylation or phosphorylation of IκB-α (Fig. 7*A*). We also compared IKK-α and IKK-α(S176E) in an NF-κB reporter gene assay in transiently transfected HeLa cells. Mutation of Ser-176 to glutamic acid significantly enhanced the ability of IKK-α to activate NF-κB (Fig. 7*B*).

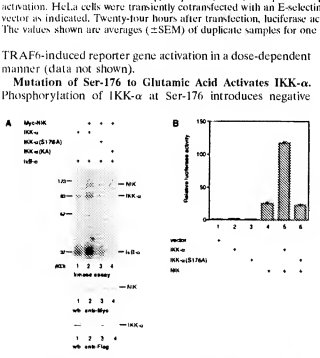

FIG. 5. Loss of IKK-α(S176A) activation by NIK. (*A*) Loss of IKK-α(S176A) activation by NIK in kinase assay. 293 cells were transiently transfected with expression plasmids for FLAG epitope-tagged IKK-α or IKK-α(S176A) and Myc-epitope-tagged NIK. IKK-α proteins (and coprecipitating Myc-NIK proteins) were purified with anti-FLAG antibodies, and *in vitro* phosphorylation reactions were carried out by using bacterially expressed IκB-α and [γ-^{32}P]ATP. The amounts of protein used were determined by immunoblotting with anti-Myc polyclonal antibodies (*Middle*), and with anti-FLAG polyclonal antibodies (*Bottom*). (*B*) Loss of IKK-α(S176A) activation by NIK in an NF-κB reporter gene assay. 293 cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α and NIK expression vectors as indicated. Thirty to 36 hr after transfection, luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages (±SEM) of duplicate samples for one representative experiment.

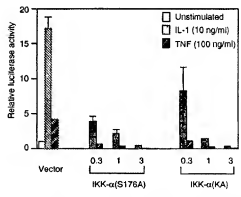

FIG. 6. IKK-α(S176A) is a dominant negative inhibitor of IL-1 and TNF-induced NF-κB activation. 293/IL-1R1 cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α(S176A) expression vector as indicated. Twenty-four hours after transfection, cells were either left untreated, or stimulated for 6 hr with IL-1 (10 ng/ml) or TNF (100 ng/ml) prior to harvest. Luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages (±SEM) of duplicate samples for one representative experiment.

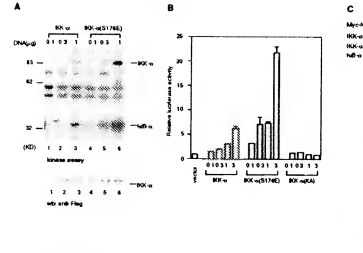

FIG. 7. IKK-α(S176E) is constitutively active. (*A*) IKK-α(S176E) has significantly greater activity than IKK-α in kinase assay. HeLa cells were transiently transfected with expression plasmids for FLAG epitope-tagged IKK-α or IKK-α(S176E) at different doses. Thirty hours later, IKK-α proteins were purified with anti-FLAG antibodies, and *in vitro* phosphorylation reactions were carried out with bacterially expressed IκB-α and [γ-^{32}P]ATP. The amounts of protein used were determined by immunoblotting with anti-FLAG antibodies (as shown in the lower panel). (*B*) IKK-α(S176E) has significantly greater activity than IKK-α in an NF-κB reporter gene assay. HeLa cells were transiently cotransfected with an E-selectin-luciferase reporter gene plasmid and vector control or IKK-α expression vector as indicated. Twenty-four hours after transfection, luciferase activities were determined and normalized on the basis of β-gal expression. The values shown are averages (±SEM) of duplicate samples for one representative experiment. (*C*) IKK-α(S176E) activity is independent of NIK activation. HeLa cells were transiently transfected with expression plasmids for FLAG epitope-tagged IKK-α or IKK-α(S176E) and Myc-epitope-tagged NIK. IKK-α proteins were purified with anti-FLAG antibodies, and *in vitro* phosphorylation reactions were carried out by using bacterially expressed IκB-α and [γ-^{32}P]ATP. The amounts of IKK-α protein used were determined by immunoblotting with anti-FLAG antibodies (*Lower*).

The IKK-α(S176E) mutant is more active than wild-type IKK-α in both kinase assay and reporter assay. However, it is still important to know whether the IKK-α(S176E) mutant can be further activated by the upstream kinase NIK. In the experiments shown in Fig. 7*C*, we expressed FLAG epitope-tagged wild-type IKK-α or the IKK-α(S176E) mutant together with Myc epitope-tagged NIK in HeLa cells. We then measured the ability of the immunopurified IKK-α to phosphorylate recombinant IκB-α. The activity of wild-type IKK-α was strongly enhanced upon activation by the coexpressed NIK kinase. In contrast, IKK-α(S176E) mutant was similarly active when expressed either with or without NIK, suggesting that IKK-α(S176E) activity is independent of upstream activation.

DISCUSSION

The TNF- and IL-1-induced NF-κB activation pathways converge at NIK, a MAP3K-related serine/threonine kinase (14). NIK forms a complex with two IKKs, IKK-α and IKK-β (16, 20). IKK-α and IKK-β are serine/threonine kinases that phosphorylate members of the IκB family on two specific serine residues in a signal-induced process that is required for IκB degradation and NF-κB activation (16–18, 20, 21). In this study, we have shown that IKK-α is activated via serine phosphorylation by the upstream kinase NIK. NIK phosphorylates IKK-α on Ser-176 that lies in the activation loop (28) between subdomains VII and VIII. Mutation of Ser-176 to alanine results in a kinase-inactive form of IKK-α that is impaired not only in phosphorylating IκB-α and activating an NF-κB reporter gene, but which also can no longer be activated by NIK. Conversely, replacement of Ser-176 with glutamic acid results in a constitutively active IKK-α whose activity in both kinase and NF-κB reporter assays is independent of upstream kinase NIK. The importance of Ser-176 is further suggested by the ability of the IKK-α(S176A) mutant to block IL-1- and TNF-induced NF-κB activation. Although IKK-α can be activated via NIK-mediated phosphorylation on Ser-176, it is possible that other kinases may exist that can also phosphorylate and activate IKK-α.

The site of the NIK-activating phosphorylation of IKK-α lies between kinase subdomains VII and VIII. This phosphorylation and activation of IKK-α by NIK is reminiscent of the MAP kinase pathway in which the upstream kinases Raf and MEKK activate MEK via phosphorylation on Ser-218 and Ser-222 between kinase subdomains VII and VIII (25–27). MEK then activates MAPK by phosphorylating its corresponding activation loop on Thr-183 and Tyr-185 (30). The crystal structure of MAP kinase demonstrates that the activation loop lies in a solvent-exposed portion of the protein and phosphorylation of residues in this region can stabilize the kinase in an active conformation (28, 29). The spatial conservation of the sites activating kinase activity suggests that this mode of regulation is strongly conserved, especially among kinases found in signal transduction cascades. Activation sites in other related kinases might thus be inferred by homology to this region.

Although IKK-α and IKK-β share 52% identity, they are differentially phosphorylated by NIK. NIK appears to phosphorylate IKK-α better than IKK-β. However, we do not know if this difference is reflected in biological differences in the signaling processes affected by these two kinases. Recent experiments suggest that IKK-α and IKK-β form a heterocomplex that interacts directly with the upstream kinase NIK (18, 20, 21). IKK-β may be an inherently better IκB-α kinase than IKK-α and therefore might not need to be further activated by NIK phosphorylation. It is also possible IKK-β may become activated by autophosphorylation when overexpressed. Alternatively, IKK-β may also require phosphorylation by IKK-α or another kinase to be activated. Mercurio *et al.* (16) recently reported that an IKK-β(SS177, 181EE) mutant is constitutively active, supporting the view that phosphorylation also plays an important role in the activation of IKK-β.

IKK-α can be activated by a variety of external stimuli (17). Although the activation mechanism of IKK-α is emerging, little is known about the IKK-α inactivation process that occurs rapidly following the activation (17). It is possible that a phosphatase specifically dephosphorylates Ser-176 and inac-

tivates IKK-α, or that additional sites on IKK-α become dephosphorylated, resulting in enzyme inactivation.

We can now fill in additional details to the pathway by which TNF binding on the cell surface results in NF-κB activation in the nucleus. TNF interaction with TNF-R1 results in receptor trimerization and subsequent association with the adaptor molecule TRADD via the death domains of both proteins. TRADD then recruits TRAF2, RIP, and other signaling molecules, resulting in the formation of the TNF-R1 signaling complex. In a step that is not yet understood and that may require RIP and/or TRAF proteins, NIK becomes activated. NIK then phosphorylates IKK-α (or the α subunit of an IKK-α/IKK-β heterodimer) on Ser-176 in the IKK-α activation loop. Once activated by NIK, IKK-α phosphorylates Ser-32 and Ser-36 of IκB-α, signaling IκB-α for degradation, and allowing NF-κB translocation to the nucleus.

We thank Keith Williamson for DNA sequencing and Catherine Regnier, Mike Rothe, John Woronicz, and Ho Yeong Song for expression vectors. We thank Patrick Baeuerle, Catherine Regnier, Lin Wu, Xiong Gao, and Csaba Lebel for helpful discussions and Patrick Baeuerle, Mike Rothe, and Vijay Baichwal for comments on the manuscript.

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(12) **United States Patent**
May et al.

(10) **Patent No.: US 6,864,355 B1**
(45) **Date of Patent: Mar. 8, 2005**

(54) **INHIBITION OF NF-κB ACTIVATION BY BLOCKADE OF IKKβ-NEMO INTERACTIONS AT THE NEMO BINDING DOMAIN**

(75) Inventors: **Michael J. May**, New Haven, CT (US); **Sankar Ghosh**, Madison, CT (US)

(73) Assignee: **Yale University**, New Haven, CT (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 320 days.

(21) Appl. No.: **09/643,260**

(22) Filed: **Aug. 22, 2000**

Related U.S. Application Data

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(51) **Int. Cl.**[7] **C07K 14/00**; C07K 16/00; A01N 37/18; A61K 38/00

(52) **U.S. Cl.** **530/300**; 530/324; 530/329; 514/2; 514/12; 514/17

(58) **Field of Search** 530/300, 324, 530/329; 514/2, 12, 17

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(57) **ABSTRACT**

The invention includes compositions and methods for the selective inhibition of cytokine-mediated NF-κB activation by blocking the interaction of NEMO with IκB kinase-β (IKKβ) at the NEMO binding domain (NBD). The blockade of IKKβ-NEMO interaction resulting in inhibition of IKKβ kinase activity and subsequent decreased phosphorylation of IκB. Phosphorylation of IκB being an integral step in cytokine-mediated NF-κB activation. The invention further includes methods for screening for agents capable of interacting at the NBD and therapeutic uses for such agents in pathological disorders caused by dysregulation of NF-κB activation.

22 Claims, 16 Drawing Sheets

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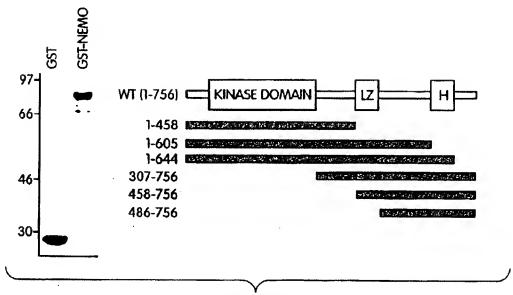
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GST-NEMO
97
66
46
30
WT (1-756)
KINASE DOMAIN
LZ
H
1-458
1-605
1-644
307-756
458-756
486-756

Fig. 1A

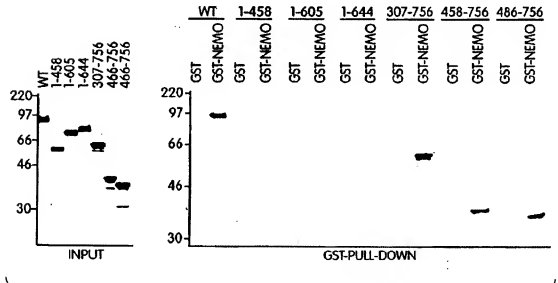
WT
1-458
1-605
1-644
307-756
458-756
486-756
GST
GST-NEMO
220
97
66
46
30
INPUT
GST-PULL-DOWN

Fig. 1B

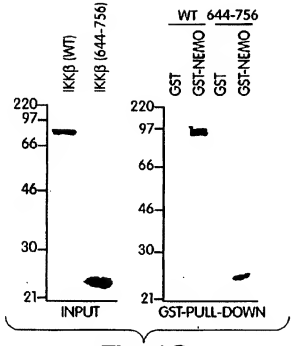
IKKβ (WT)
IKKβ (644-756)
WT
644-756
GST
GST-NEMO
GST
GST-NEMO
220
97
66
46
30
21
INPUT
GST-PULL-DOWN

Fig. 1C

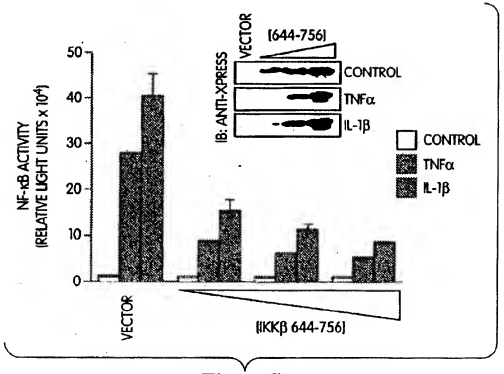
NF-κB ACTIVITY
(RELATIVE LIGHT UNITS x 10⁴)
50
40
30
20
10
0
VECTOR
[IKKβ 644-756]
IB: ANTI-XPRESS
VECTOR
[644-756]
CONTROL
TNFα
IL-1β
CONTROL
TNFα
IL-1β

Fig. 1D

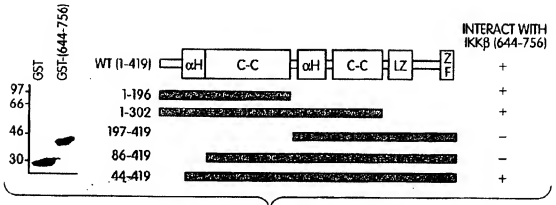

Fig. 2A

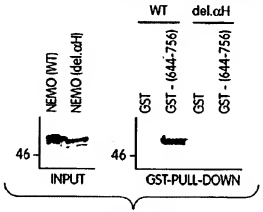

Fig. 2B

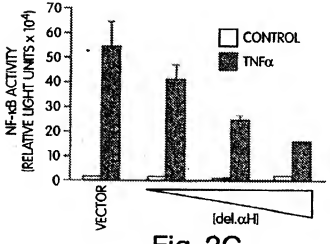

Fig. 2C

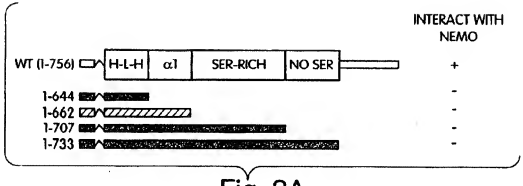
INTERACT WITH NEMO
WT (1-756)
H-L-H
α1
SER-RICH
NO SER
+
1-644
-
1-662
-
1-707
-
1-733
-

Fig. 3A

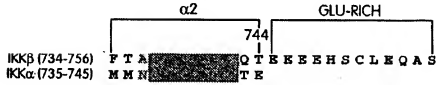
α2
GLU-RICH
744
IKKβ (734-756) F T A Q T E E E E H S C L E Q A S
IKKα (735-745) M M N T E

Fig. 3B

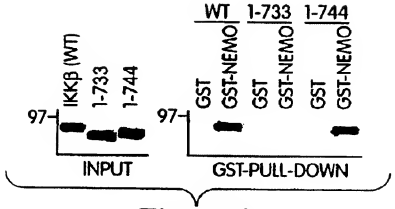
WT
1-733
1-744
IKKβ (WT)
1-733
1-744
GST
GST-NEMO
GST
GST-NEMO
GST
GST-NEMO
97
97
INPUT
GST-PULL-DOWN

Fig. 3C

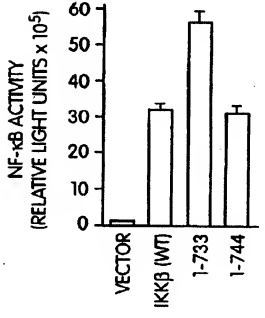
NF-κB ACTIVITY
(RELATIVE LIGHT UNITS x 10⁵)
60
50
40
30
20
10
0
VECTOR
IKKβ (WT)
1-733
1-744

Fig. 3D

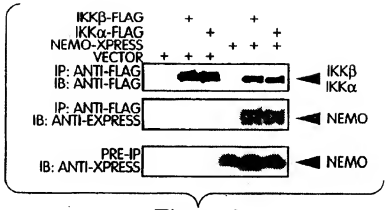

Fig. 4A

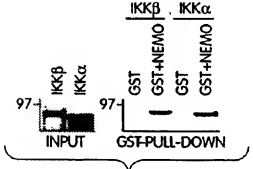

Fig. 4B

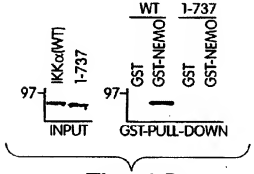

Fig. 4C

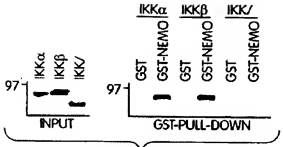

Fig. 4D

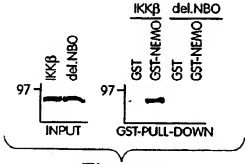

Fig. 4E

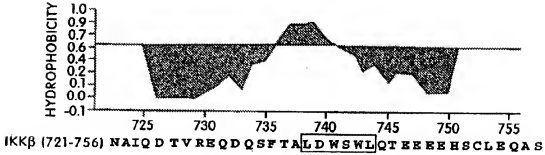

Fig. 4F

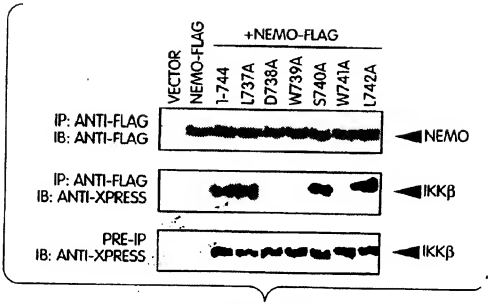
+NEMO-FLAG
VECTOR
NEMO-FLAG
1-744
L737A
D738A
W739A
S740A
W741A
L742A
IP: ANTI-FLAG
IB: ANTI-FLAG
NEMO
IP: ANTI-FLAG
IB: ANTI-XPRESS
IKKβ
PRE-IP
IB: ANTI-XPRESS
IKKβ

Fig. 4G

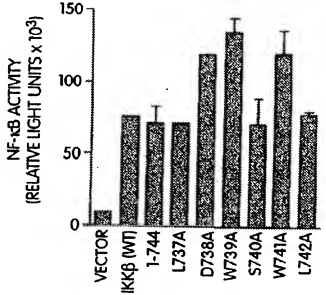
NF-κB ACTIVITY
(RELATIVE LIGHT UNITS x 10³)
150
100
50
0
VECTOR
IKKβ (WT)
1-744
L737A
D738A
W739A
S740A
W741A
L742A

Fig. 4H

WILD-TYPE: drqikiwfqnrrmkwkkTALDWSWLQTE

MUTANT: drqikiwfqnrrmkwkkTALDASALQTE

Fig. 5A

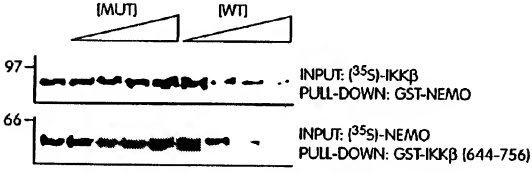

Fig. 5B

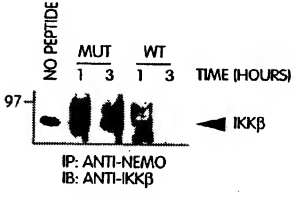

Fig. 5C

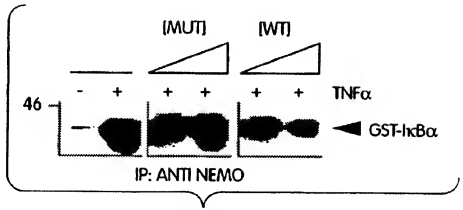
[MUT]
[WT]
- + + + + + TNFα
46
GST-IκBα
IP: ANTI NEMO

Fig. 5D

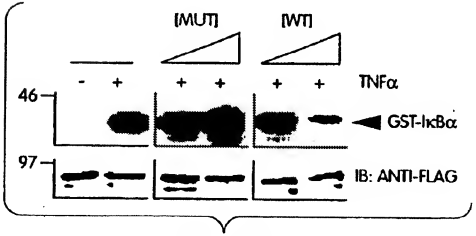
[MUT]
[WT]
- + + + + + TNFα
46
GST-IκBα
97
IB: ANTI-FLAG

Fig. 5E

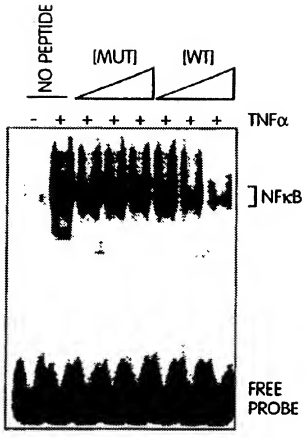
NO PEPTIDE
[MUT]
[WT]
- + + + + + + +
TNFα
NFκB
FREE
PROBE

Fig. 5F

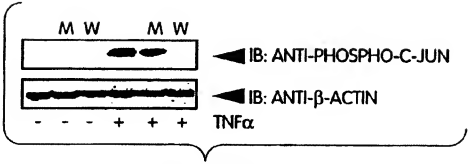
M W M W
IB: ANTI-PHOSPHO-C-JUN
IB: ANTI-β-ACTIN
- - - + + +
TNFα

Fig. 5G

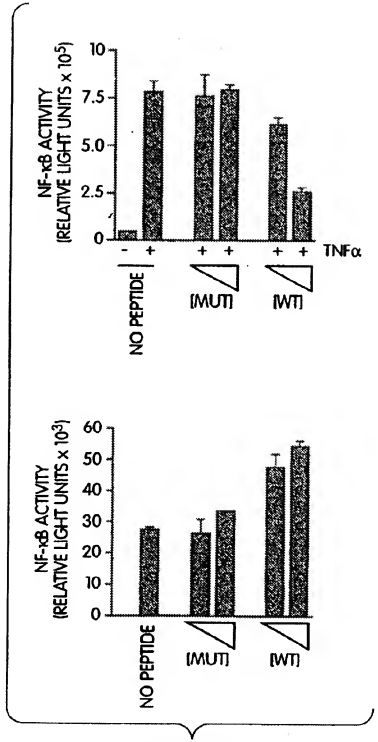
NF-κB ACTIVITY
(RELATIVE LIGHT UNITS x 10⁵)
10
7.5
5.0
2.5
0
- +
+ +
+ +
TNFα
NO PEPTIDE
[MUT]
[WT]
NF-κB ACTIVITY
(RELATIVE LIGHT UNITS x 10³)
60
50
40
30
20
10
0
NO PEPTIDE
[MUT]
[WT]

Fig. 5H

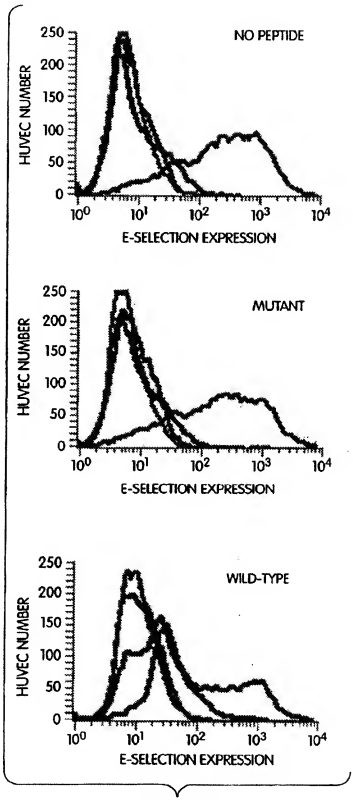
NO PEPTIDE
HUVEC NUMBER
250
200
150
100
50
0
10^0 10^1 10^2 10^3 10^4
E-SELECTION EXPRESSION
MUTANT
HUVEC NUMBER
250
200
150
100
50
0
10^0 10^1 10^2 10^3 10^4
E-SELECTION EXPRESSION
WILD-TYPE
HUVEC NUMBER
250
200
150
100
50
0
10^0 10^1 10^2 10^3 10^4
E-SELECTION EXPRESSION

Fig. 6A

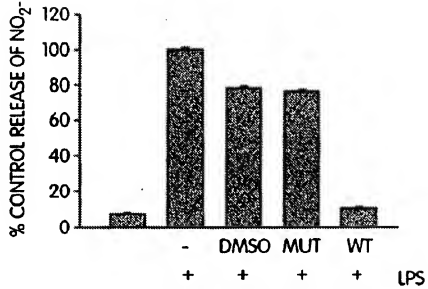
% CONTROL RELEASE OF NO_2^-
120
100
80
60
40
20
0
- DMSO MUT WT
+ + + + LPS

Fig. 6B

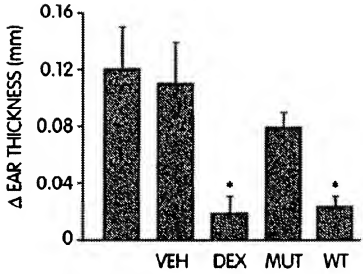
Δ EAR THICKNESS (mm)
0.16
0.12
0.08
0.04
0
*
*
VEH DEX MUT WT

Fig. 6C

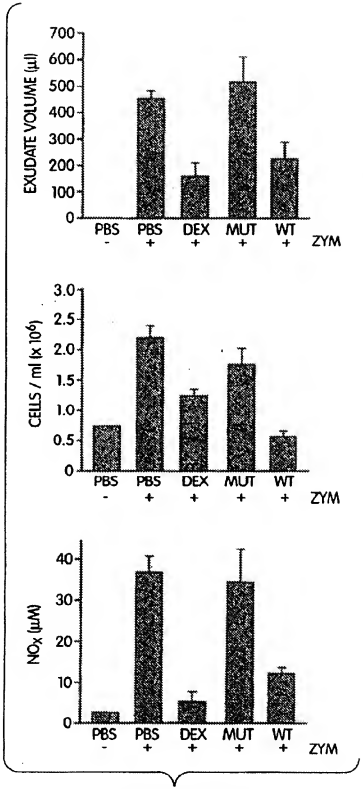
EXUDATE VOLUME (µl)
700
600
500
400
300
200
100
0
PBS -
PBS +
DEX +
MUT +
WT +
ZYM
CELLS / ml (x 10⁶)
3.0
2.5
2.0
1.5
1.0
0.5
0
PBS -
PBS +
DEX +
MUT +
WT +
ZYM
NOx (µM)
40
30
20
10
0
PBS -
PBS +
DEX +
MUT +
WT +
ZYM

Fig. 6D

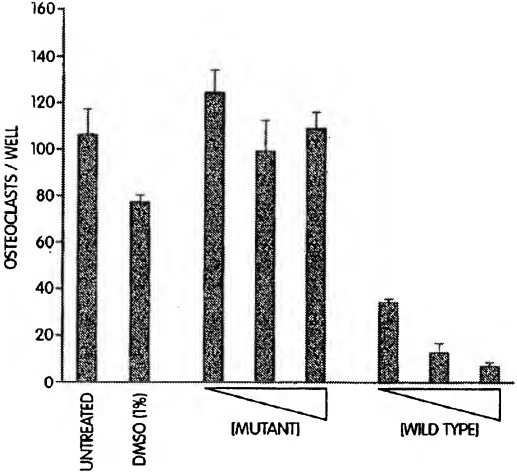
160
140
120
100
80
60
40
20
0
OSTEOCLASTS / WELL
UNTREATED
DMSO (1%)
[MUTANT]
[WILD TYPE]

Fig. 7

INHIBITION OF NF-κB ACTIVATION BY BLOCKADE OF IKKβ-NEMO INTERACTIONS AT THE NEMO BINDING DOMAIN

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application Ser. No. 60/201,261 filed May 2, 2000 which is herein incorporated by reference in its entirety.

U.S. GOVERNMENT SUPPORT

This work was supported by a grant from the National Institute of Health (AI33443).

FIELD OF THE INVENTION

The invention relates to compositions and methods for the selective inhibition of cytokine-mediated NF-κB activation by blocking the interaction of NEMO with IκB kinase-β (IKKβ) at the NEMO binding domain (NBD). The blockade of IKKβ-NEMO interaction results in inhibition of IKKβ kinase activation and subsequent decreased phosphorylation of IκB. Phosphorylation of IκB is an integral step in cytokine-mediated NF-κB activation.

BACKGROUND OF THE INVENTION

NF-κB is a transcription factor which mediates extracellular signals responsible for induction of genes involved in pro-inflammatory responses (see Baltimore et al., (1998) U.S. Pat. No. 5,804,374). NF-κB is anchored in the cytoplasm of most non-stimulated cells by a non-covalent interaction with one of several inhibitory proteins known as IκBs (see May & Ghosh., (1997) Semin. Cancer. Biol. 8, 63–73; May & Ghosh, (1998) Immunol. Today 19, 80–88; Ghosh et al., (1998) Annu. Rev. Immunol. 16, 225–260). Cellular stimuli associated with pro-inflammatory responses such as TNFα, activate kinases, which in turn activate NF-κB by phosphorylating IκBs. The kinases that phosphorylate IκBs are called IκB kinases (IKKs).

Phosphorylation targets IκBs for ubiquitination and degradation. The degradation and subsequent dissociation of IκBs from NF-κB reveals the nuclear localization signal on NF-κB, resulting in nuclear translocation of active NF-κB, leading to up-regulation of genes responsive to NF-κB (May & Ghosh, (1997) Semin. Cancer. Biol. 8, 63–73; May & Ghosh, (1998) Immunol. Today 19, 80–88; Ghosh et al., (1998) Annu. Rev. Immunol. 16, 225–260; Siebenlist et al., (1994) Annu. Rev. Cell Biol. 12, 405–455). Phosphorylation of IκBs is therefore an essential step in the regulation of NF-κB mediated pro-inflammatory responses.

The identification and characterization of kinases that phosphorylate IκBs have led to a better understanding of signaling pathways involving NF-κB activation. Several different subtypes of IKK have been identified thus far. IKKα was initially identified as an IκB kinase induced by TNFα stimulation in HeLa cells (DiDonato et al., (1997) Nature 388, 548–554). Another IκB kinase homologous to IKKα was identified, termed IKKβ and determined to be the major IκB kinase induced following TNFα stimulation (Takeda et al., (1999) Science 284, 313–316; Hu et al., (1999) Science 284, 316–320; Li et al., (1999) Science 284, 321–325; Pot et al., (2000) U.S. Pat. No. 6,030,834; Woronicz & Goeddel (1999) U.S. Pat. No. 5,939,302). IKKα and IKKβ have 52% overall homology and 65% homology in the kinase domain (Zandi et al., (1997) Cell 91, 243–252).

IκB protein kinases (IKKs) phosphorylate IκBs at specific serine residues. For example, they specifically phosphorylate serines 32 and 36 of IκBα (Traenckner et al., (1995) EMBO J. 14, 2876–2883; DiDonato et al., (1996) Mol. Cell. Biol. 16, 1295–1304). Phosphorylation of both sites is required to efficiently target IκBα for degradation. Furthermore, activation of IKKα and IKKβ is usually in response to NF-κB activating agents and mutant IKKα and IKKβ, which are catalytically inactive, can be used to block NF-κB stimulation by cytokines such as TNFα and IL-1 (Régnier et al., (1997) Cell 90, 373–383; Delhase et al., (1999) Science 284, 309–313). IκB protein kinases are therefore essential in regulation of NF-κB activation processes.

IKKα and IKKβ have distinct structural motifs including an amino terminal serine-threonine kinase domain separated from a carboxyl proximal helix-loop-helix (H-L-H) domain by a leucine zipper domain. These structural characteristics are unlike other kinases, and the non-catalytic domains are thought to be involved in protein-protein interactions. Proteins which bind to IKKs may therefore be capable of regulating the activity of NF-κB (Marcu et al., (1999) U.S. Pat. No. 5,972,655) and potentially regulating downstream events such as induction of NF-κB. For instance, NEMO (NF-κB Essential Modulator) is a protein which has been identified to bind to IKKs and facilitate A kinase activity (Yamaoke et al., (1998) Cell 93, 1231–1240; Rothwarf et al., (1998) Nature 395, 287–300; Mercurio et al., (1999) Mol. Cell. Biol. 19, 1526–1538; Haraj & Sun, (1999) J. Biol. Chem. 274, 22911–22914; Jin & Jcang, (1999) J. Biomed. Sci. 6, 115–120).

The discovery of agents capable of modulating IKK binding proteins involved in the regulation of NF-κB induction will be useful for controlling NF-κB mediated pro-inflammatory processes. A particular advantage of such agents is that while blocking NF-κB induction via IKK, they would not inhibit the basal activity of NF-κB. This invention provides for the identification and characterization of agents which modulate IKK binding proteins such as the NF-κB Essential Modulator (NEMO) protein.

SUMMARY OF THE INVENTION

This invention provides compositions and methods for the selective inhibition of cytokine-mediated NF-κB activation by blocking the interaction of NEMO with IκB kinase-β (IKKβ) at the NEMO binding domain (NBD).

In one aspect, the present invention provides methods of inhibiting NF-κB induction in a cell which includes administering an effective amount of a peptide which blocks the interaction of one or more IKKs and NEMO. In one specific embodiment, this invention includes such methods wherein the IKKs are IKKα and/or IKKβ. In another specific embodiment of this invention, the peptides include at least one NEMO binding domain. In still another specific embodiment of this invention, such methods utilize peptides which include at least one membrane translocation domain. In still another specific embodiment of this invention, the peptides utilized in such methods include amino acid sequences comprising the sequences of SEQ ID NO: 2, 4, 5, 6, 11, 12, 16, 17 or 18.

In another aspect, the present invention provides methods of inhibiting inflammation in a mammal by administering an effective amount of a peptide which blocks the interaction of IKK and NEMO. In one specific embodiment, such methods include peptides which block the recruitment of leukocytes into sites of acute and chronic inflammation. In another specific embodiment, the methods of the present invention include peptides which down-regulate the expression of

E-selectin on leukocytes. In yet another embodiment, the methods include peptides which block osteoclast differentiation.

In another aspect, the present invention provides methods of inhibiting NF-κB-dependent target gene expression in cells by administering effective amounts of a peptide which block the interaction of one or more IKKs and NEMO. In one specific embodiment, this invention provides such methods wherein the IKK is IKKβ. In another specific embodiment, this invention provides such methods wherein the NF-κB-dependent target-gene is E-selectin.

In another aspect, the present invention provides methods of identifying agents which interact with the NEMO binding domain wherein the methods include the steps of:

(a) exposing cells which express NEMO and NF-κB to the agents; and

(b) determining whether the agents modulate activation of NF-κB by the cell, wherein an alteration in activation of NF-κB is indicative of agents which interact with the NEMO binding domain.

In another aspect, the present invention provides methods of identifying agents which modulate the activity of NEMO which includes the steps of:

(a) exposing cells which express NEMO to the agents; and

(b) determining whether the agents modulate the activity of NEMO, thereby identifying agents which modulate the activity of NEMO.

In another aspect, the present invention provides fusion peptides which include the NEMO binding domain and at least one membrane translocation domain. In a preferred embodiment, the fusion peptide membrane translocation domain facilitates membrane translocation in vivo. In a specific embodiment, this invention provides such fusion peptides wherein the membrane translocation domain is either the third helix of the antennapedia homeodomain or the HIV-1 Tat protein. In another specific embodiment, this invention provides such fusion peptides wherein the NEMO binding domain is SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19. In still another aspect, this invention provides compositions which include such fusion peptides. In yet another embodiment, this invention provides such compositions which also include one or more carriers.

In another aspect, the present invention provides isolated peptides such as the following:

(a) an isolated peptide which includes the amino acid sequence of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or19

(b) an isolated peptide which includes a fragment of at least three amino acids of an amino acid sequence of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19;

(c) an isolated peptide which includes conservative amino acid substitutions of the amino acid sequences of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19; and

(d) naturally occurring amino acid sequence variants of amino acid sequences of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19. In still another aspect, this invention provides compositions which include such isolated peptides. In yet another embodiment, this invention provides such compositions which also include one or more carriers.

In another aspect, this invention provides isolated peptides which consist of the amino acid sequences of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and/or 19.

DESCRIPTION OF THE DRAWINGS

FIG. 1—NEMO interacts with the COOH-terminus of IKKβ. (A) GST alone or GST-NEMO were precipitated from bacterial lysates using glutathione-agarose, separated by SDS-PAGE (10%) and the gel was stained with Coomassie blue (left panel). Equal amounts of GST or GST-NEMO were used in subsequent GST pull-down experiments. The scheme depicted in the right panel represents the COOH- and NR_2-terminal truncation mutants of IKKβ used to determine the region of NEMO interaction. (B) IKKβ muants were cloned, expressed by in vitro translation (input; left panel) and used for GST pull-down (right panel). (C) Wild-type IKKβ and IKKβ-(644–756) were in vitro translated (left panel) and used for GST pull-down analysis (left panel): (D) HeLa cells were transiently transfected with either vector alone or increasing concentrations (0.25, 0.5, 1.0 μg/ml) of the xpress-tagged IKKβ-(644–756) construct together with the pBIIX-luciferase reporter plasmid. After forty-eight hours cells were treated with either TNFα (10 ng/ml) or IL-1β (10 ng/ml) for four hours then NF-κB activity was measured. Western blot analysis from portions of the lysate using anti-xpress (inset) demonstrates the increasing levels of expressed protein.

FIG. 2—The first α-helical region of NEMO is required for binding to IKKβ. (A) A truncated version of IKKβ consisting of only the COOH-terminus from residue V644 to S756 was fused with GST (GST-644–756) and expressed in bacteria. After precipitation by glutathione agarose, GST alone and GST-(644–756) were separated by SDS-PAGE (10%) and the gel was stained with Coomassie blue (left panel). Equal amounts of each protein were used for subsequent GST pull-down analyses. Various NH_2- and COOH-terminal truncations of NEMO were constructed, [^{35}S]-methionine labeled and used for in vitro pull down (right panel). Mutants that interacted with GST-(644–756) are indicated (+). None of the mutants interacted with GST alone. (B) Wild-type NEMO and a deletion mutant lacking the first α-helical region (del.αH) were in vitro translated (left panel: input) and used for GST pull-down using the proteins shown above (A: left). (C) HeLa cells were transfected with pBIIx-luciferase together with either pcDNA-3 (vector) or increasing concentrations of del.αH (0.25, 0.5, 1.0 μg/ml) for forty-eight hours then treated for four hours with TNFα (10 ng/ml). Cells were then lysed and NF-κB activity was measured by luciferase assay.

FIG. 3—Interaction with NEMO and functional kinase activity requires an IKKα-homologous region of the IKKβ COOH-terminus. (A) Truncation mutations of IKKβ sequentially omitting the extreme COOH-terminus (1–733), the serine-free region (1–707), the serine rich-domain (1–662) and the α_1-region (1–644) were expressed and labeled by in vitro translation and used for GST pull-down by (GST-NEMO (FIG. 1A). None of the mutants interacted with GST alone. (B) Sequence alignment of the extreme COOH-termini of IKKβ and IKKα. The α_1- and glutamate-rich regions are indicated and the six identical amino acids are shaded. (C) Wild-type IKKβ and the truncation mutants (1–733 and 1–744) were [^{35}S]-methionine-labeled (input) and used for in vitro pull down with either GST alone or GST-NEMO. (D) HeLa cells were transfected for 48 hours with 1 μg/ml of the indicated constructs or empty vector (pcDNA-3) together with pHIx-luciferase. NF-κB activity was determined by luciferase assay.

FIG. 4—Association of NEMO with IKKβ and IKKα reveals the NEMO binding domain (NBD) to be six COOH-terminal amino acids. (A) COS cells transiently transfected

with vector alone, FLAG-tagged IKKα or IKKβ (1 μg/well) or xpress-tagged NEMO (1 μg/well) to a total DNA concentration of 2 μg/well as indicated. Following lysis, immunoprecipitations (IP) were performed using anti-FLAG (M2) and the contents of precipitates visualized by immunoblotting (IB) with either anti-FLAG (M2) or anti-xpress. A portion of pre-IP lysate was immunoblotted with anti-xpress to ensure equivalent levels of NEMO expression in transfected cells. (B) NEMO interacted equally well with both IKKβ and IKKα. (C) Wild-type IKKα and IKKα-(1–737) were expressed and labeled (input) and used for GST pull-down using GST or GST-NEMO. (D) Full length cDNA encoding human IKK_γ was obtained by RT-PCR from HeLa cell mRNA using the Expand™ Long Template PCR System (Boehringer Mannheim), the forward primer (5'-CTAGTCGAATTCACCATGCAGAGCACAGCCAATTAC) (SEQ ID NO: 22) and the reverse primer (3'-CTAGTCTCTAGATTAGACATCAGGAGGTGCTGG) (SEQ ID NO: 23) and cloned into the EcoRI and XbaI sites of pcDNA-3. GST pull-down analysis was performed using [^{35}S]-methionine-labeled IKKα, IKKβ and IKK_γ. (E) A deletion mutant of IKKβ lacking the NBD (delNBD) was [^{35}S]-methionine-labeled (input) and used for GST pull down analysis. (F) A Fauchere-Pliska hydrophobicity plot of the COOH-terminus (5721–5756) of human IKKβ was generated using MacVector™ (version 6.5.3) software. The NBD (L737–L742) is boxed. (G) COS cells were transfected for forty-eight hours with a total of 2 μg DNA/well of either vector alone, vector plus NEMO-FLAG or NEMO-FLAG plus xpress-tagged versions of IKKβ-(1–744) containing point mutations within the NBD as indicated. Following lysis and immunoprecipitation using anti-FLAG (M2), immunoblot analysis was performed with either anti-FLAG or anti-xpress. The level of expressed protein in pre-IP lysate was determined by immunoblotting with anti-xpress (lower panel). (H) HeLa cells were transiently transfected for forty-eight hours with the indicated constructs together with pBIIX-luciferase and NFκB activity in lysate was measured by luciferase assay.

FIG. **5**—A cell-permeable peptide spanning the IKKβ NBD inhibits the IKKβ/NEMO interaction, TNFα-induced NF-κB activation and NF-κB-dependent gene expression. (A) Sequences of wild-type and mutant forms of IKKβ NBD peptide. (B) GST-pull-down analysis was performed using either GST-NEMO-in vitro translated IKKβ (upper panel) or GST-IKKβ-(644–756)-in vitro translated NEMO (lower panel). The assay was performed in the absence (no peptide) or presence of increasing concentrations (125, 250, 500 or 1000 μM) of either mutant (MUT) or wild-type (WT) NBD peptide. (C) HeLa cells were incubated with either peptide (200 μM) for the times indicated. Following lysis, the IKK complex was immunoprecipitated using anti-NEMO and the resulting immunoblot probed with anti-IKKβ. (D) Gel image showing anti-NEMO immunoprecipitation. (E) Gel image showing anti-FLAG immunoblot. (F) HeLa cells were incubated for three hours with increasing concentrations (50, 100 or 200 μM) of each peptide followed by treatment for fifteen minutes with TNFα (10 ng/ml) as indicated (+). Following lysis, nuclear extracts were made and 10 μg of protein from each sample was used for EMSA using a specific [^{32}P]-labeled κB-site probe. (G) Gel image showing anti-Phospho-C-Jun immunoblot and anti-β-Actin immunoblot. (H) HeLa cells were transfected for forty-eight hours with pBIIX-luciferase then incubated for two hours in the absence (control) or presence of mutant or wild-type NBD peptide (100 and 200 μM of each). Subsequently the cells were either treated with TNFα (10 ng/ml) as indicated (top panel) or left untreated (bottom panel) for a further four hours after which NF-κB activation was measured by luciferase assay.

FIG. **6**—The wild-type NBD peptide inhibits NF-κB-induced gene expression and experimentally induced inflammation. (A) Primary HUVEC were pre-incubated for two hours with wild-type (middle) or mutant (bottom) NBD peptides (100 μM) then stimulated with TNFα (10 ng/ml) for a further six hours. Control cells received no peptide. Cells were stained with either anti-E-selectin (H4/18) or a non-binding control antibody (K16/16) and expression was measured by FACS (FACSort, Becton Dickinson). The profiles show E-selectin staining in the absence (shaded) and presence (solid line) of TNFα and control antibody staining under the same conditions (dashed line, no TNFα; dotted line, TNFα). (B) % control release of NO_2- in various samples. (C) PMA-induced ear edema in mice topically treated with either vehicle (VEH), dexamethasone (DEX) or NBD peptides was induced and measured as described in Example 8. Data are presented as mean differences in ear thickness±SD (* =p<0.05 compared with both untreated control [–] and vehicle [VEH]). (D) The effects of the NBD peptide compared with the effect of dexamethasone (DEX) on Zymosan (ZYM)-induced peritonitis in mice were determined as described again in Example 8. Control mice were injected with phosphate-buffered saline (PBS).

FIG. **7**—Dose dependent inhibition of osteoclast differentiation by wild-type but not mutant NBD peptides. Data are presented as the mean determination of triplicate samples ±SD.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

I. General Description

The invention relates generally to compositions and methods for regulation of cytokine-induced NF-κB activation associated with pro-inflammatory responses. Specifically, the invention relates to compositions and methods for blocking the interaction of NEMO with IKKβ at the NEMO binding domain (NBD), thereby inhibiting phosphorylation, degradation and subsequent dissociation of IκB from NF-κB. This inhibition results in blockade of NF-κB activation associated with pro-inflammatory responses.

The invention also includes methods for screening and identifying agents capable of interacting with the NBD thereby blocking the interaction of NEMO with IKKβ. The invention further includes the use of these agents in the treatment of disorders associated with regulation of NF-κB induction.

II. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, the term "binding" refers to the adherence of molecules to one another, such as, but not limited to, enzymes to substrates, antibodies to antigens, DNA strands to their complementary strands. Binding occurs because the shape and chemical nature of parts of the molecule surfaces are "complementary". A common metaphor is the "lock-and-key" used to describe how enzymes fit around their substrate.

The term "fusion peptide" or "fusion polypeptide" or "fusion protein" refers to a peptide, polypeptide or protein that its obtained by combining two distinct amino acid sequences. Typically, a partial sequence from one peptide, polypeptide or protein is linked to another heterologous peptide, polypeptide or protein, using cDNA technology.

The terms "conservative variation" or "conservative substitution" as used herein refers to the replacement of an amino acid residue by another, biologically similar residue. Conservative variations or substitutions are not likely to change the shape of the peptide chain. Examples of conservative variations, or substitutions, include the replacement of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine, and the like.

As used herein, the term "domain" refers to a part of a molecule or structure that shares common physicochemical features, such as, but not limited to, hydrophobic, polar, globular and helical domains or properties. Specific examples of binding domains include, but are not limited to, DNA binding domains and ATP binding domains.

As used herein, the term "membrane translocation domain" refers to a peptide capable of permeating the membrane of a cell and which is used to transport attached peptides into a cell in vivo. Membrane translocation domains include, but are not limited to, the third helix of the antennapedia homeodomain protein (Derossi et al., (1994) J. Biol. Chem. 269, 10444–10450; U.S. Pat. No. 5,888,762; U.S. Pat. No. 6,015,787) and the HIV-1 protein Tat (Lindgren et al., (2000) Trends Pharmacol. Sci. 21, 99–103).

As used herein, the term "IκB" (I kappa B) refers to any one of several members of a family of structurally related inhibitory proteins that function in the regulation of NF-κB induction.

As used herein, the term "IκB-kinase" or "IκB protein kinase" or "IκB-kinase complex" or "IκB protein kinase complex" or "IKK" refers to a kinase that phosphorylates IκBs.

As used herein, the term "IKKα" refers to the α subunit of an IκB-kinase complex.

As used herein, the term "IKKβ" refers to the β subunit of an IκB-kinase complex.

As used herein, the term "NEMO" (NF-κB Essential Modulator), "IKKγ" or "IKKAP" refers to the protein which binds to IKKs and facilitates kinase activity.

As used herein, the term "NEMO Binding Domain" or "NBD" refers to the α_2-region (residues 737–742) of wild-type IKKβ, or the corresponding six amino acid sequence of wild-type IKKα (residues 738–743) which are critical for interaction with NEMO. The nucleic acid sequence and the corresponding amino acid sequence of the wild-type NBD are provided in SEQ ID NO: 1 (GenBank Accession No. AR067807; nucleotides 2203–2235) and SEQ ID NO: 2, respectively.

As used herein, a "peptide mimetic" of a known polypeptide refers to a compound that mimics the activity of the peptide or polypeptide, but which is composed of a molecules other than, or in addition to, amino acids.

As used herein, the term "wild-type" refers to the genotype and phenotype that is characteristic of most of the members of a species occurring naturally and contrasting with the genotype and phenotype of a mutant.

III. Specific Embodiments

A. Agents which Block NEMO Interaction with IKKβ

The identification of the NBD on IKKβ allows for the discovery of compounds that are capable of down-regulating the activity of IKKβ, for example, modulating its interaction with the protein NEMO. Molecules that down-regulate interaction of NEMO with the IKK complex are therefore part of the invention. Down-regulation is defined here as a decrease in activation, function or synthesis of NEMO, its ligands or activators. It is further defined to include an increase in the degradation of the NEMO gene, its protein product, ligands or activators. Down-regulation is therefore achieved in a number of ways. For example, administration of molecules that can destabilize the binding of NEMO to IKKβ. Such molecules encompass peptide products, including those encoded by the DNA sequences of the NEMO gene or DNA sequences containing various mutations. These mutations may be point mutations, insertions, deletions or spliced variants of the IKKβ gene encoding the NBD. This invention also includes truncated peptides encoded by the DNA molecules described above. These peptides being capable of interfering with interaction of NEMO and IKKβ.

Molecules that block the phosphorylation of IκB and subsequent degradation of this protein can also be used to down-regulate NF-κB functions and are within the scope of the invention. Phosphorylation of IκB by IKKβ results in ubiquitination and degradation of IκB and subsequent dissociation of IκB, allowing for nuclear translocation of NF-κB, leading to up-regulation of genes critical to the inflammatory response. IKKβ inhibitors which interact at the NBD may therefore be used to down-regulate NF-κB function. Down-regulation of NF-κB may also be accomplished by the use of polygonal or monoclonal antibodies or fragments thereof directed against the NBD on IKKβ. Such molecules are within the claimed invention. This invention further includes small molecules with the three-dimensional structure necessary to bind with sufficient affinity at the NBD to block NEMO interactions with IKKβ. IKKβ blockade resulting in decreased degradation of IκB and decreased activation of NF-κB make these small molecules useful as therapeutic agents in blocking inflammation.

The invention further provides binding agents specific to NEMO, capable of blocking interaction of NEMO at the NBD on IKKβ. Such agents include substrates, agonists, antagonists, natural intracellular binding targets, etc. The invention also provides methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, specific binding agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving the subject proteins, e.g. NF-κB activation. Novel NEMO-specific binding agents include peptides comprising the NBD of IKKβ (residues 737–742), NEMO-specific receptors, such as somatically recombined peptide receptors like specific antibodies or T-cell antigen receptors (see Harlow & Lane, (1988) Antibodies—A Laboratory Manual, Cold Spring Harbor Laboratory Press) and other natural intracellular binding agents identified with assays such as one, two and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. Agents of particular interest modulate IKKβ function, e g. IKKβ-dependent transcriptional activation of NF-κB. For example, inhibitors of IKKβ activity may be used to regulate signal transduction involving IκB. Exemplary IKKβ inhibitors include competitive inhibitors of NEMO binding at the NBD, for example, the

peptide set forth in SEQ ID NO: 2 and conservative substitutions thereof. Conservative substitutions of amino acid residues of the peptide set forth in SEQ ID NO: 2 at positions 737, 740 and 742 are also encompassed in the invention (see Table 1 for examples of conservative substitutions which have no significant effect on NEMO binding at NBD).

Accordingly, the invention provides methods for modulating signal transduction involving IκB in a cell comprising the step of modulating IKKβ activity, e.g. by contacting the cell with an agent which inhibits the interaction of NEMO with IKKβ at the NBD, thereby inhibiting IKKβ function. The cell may reside in culture or in situ, i.e. within the natural host. Preferred inhibitors are orally active in mammalian hosts. For diagnostic uses, the inhibitors or other IKKβ binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent.

The agents discussed above represent various effective therapeutic compounds in blocking NEMO-IKKβ interactions and thus regulating NF-κB-mediated processes. Applicants have thus provided antagonists and methods of identifying antagonists that are capable of down-regulating IKKβ.

B. Screening Assays

In addition, this invention also provides compounds and methods of screening for compounds that block the function, prevent the synthesis or reduce the biologic stability of IKKβ by interacting at the NBD. Biologic stability is a measure of the time between the synthesis of the molecule and its degradation. For example, the stability of a protein, peptide or peptide mimetic (Kauvar, Nature Biotech. (1996) 14, 709) therapeutic may be shortened by altering its sequence to make it more susceptible to enzymatic degradation.

The present invention also includes methods of screening for agents which deactivate, or act as antagonists of IKKβ function. Such agents may be useful in the modulation of pathological conditions associated with alterations in IKKβ or NF-κB protein levels.

Another embodiment of the present invention provides methods for use in isolating and identifying binding partners of proteins of the invention. For example, agents which interact with IKKβ at the NBD, or interact with NEMO, thereby blocking NEMO interaction with IKKβ. In detail, a protein of the invention is mixed with a potential binding partner or an extract or fraction of a cell under conditions that allow the association of potential binding partners with the protein of the invention. After mixing, peptides, polypeptides, proteins or other molecules that have become associated with a protein of the invention are separated from the mixture. The binding partner bound to the protein of the invention can then be removed and further analyzed. To identify and isolate a binding partner, the entire protein, for instance the entire IKKβ peptide can be used. Alternatively, a fragment of the protein can be used. For example, the peptide fragment comprising NBD can be used to block interaction of IKKβ with NEMO.

A variety of methods can be used to obtain an extract of a cell. Cells can be disrupted using either physical or chemical disruption methods. Examples of physical disruption methods include, but are not limited to, sonication and mechanical shearing. Examples of chemical lysis methods include, but are not limited to, detergent lysis and enzyme lysis. A skilled artisan can readily adapt methods for preparing cellular extracts in order to obtain extracts for use in the present methods.

Once an extract of a cell is prepared, the extract is mixed with either IKKβ or NEMO under conditions in which association of the protein with the binding partner can occur. A variety of conditions can be used, the most preferred being conditions that closely resemble conditions found in the cytoplasm of a human cell. Features such as osmolarity, pH, temperature, and the concentration of cellular extract used, can be varied to optimize the association of the protein with the binding partner.

After mixing under appropriate conditions, the bound complex is separated from the mixture. A variety of techniques can be utilized to separate the mixture. For example, antibodies specific to a protein of the invention can be used to immunoprecipitate the binding partner complex. Alternatively, standard chemical separation techniques such as chromatography and density-sediment centrifugation can be used.

After removal of non-associated cellular constituents found in the extract, the binding partner can be dissociated from the complex using conventional methods. For example, dissociation can be accomplished by altering the salt concentration or pH of the mixture.

To aid in separating associated binding partner pairs from the mixed extract, the protein of the invention can be immobilized on a solid support. For example, the protein can be attached to a nitrocellulose matrix or acrylic beads. Attachment of the protein to a solid support aids in separating peptide-binding partner pairs from other constituents found in the extract. The identified binding partners can be either a single protein or a complex made up of two or more proteins. Alternatively, binding partners may be identified using a Far-Western assay according to the procedures of Takayama et al., (1997) Methods Mol. Biol. 69, 171–184 or Sauder et al., (1996) J. Gen. Virol. 77, 991–996 or identified through the use of epitope tagged proteins or GST fusion proteins.

Alternatively, the nucleic acid molecules encoding the peptides of the invention can be used in a yeast two-hybrid system. The yeast two-hybrid system has been used to identify other protein partner pairs and can readily be adapted to employ the nucleic acid molecules herein described (see for example, Stratagene Hybrizap® two-hybrid system).

Another embodiment of the present invention provides methods for identifying agents that modulate at least one activity of NEMO or IKKβ. Such methods or assays may utilize any means of monitoring or detecting the desired activity.

In one format, the relative amounts of a protein of the invention between a cell population that has been exposed to the agent to be tested compared to an un-exposed control cell population may be assayed. In this format, probes such as specific antibodies are used to monitor the differential expression of the protein in the different cell populations. Cell lines or populations are exposed to the agent to be tested under appropriate conditions and time. Cellular lysate may be prepared from the exposed cell line or population and a control, unexposed cell line or population. The cellular lysate are then analyzed with the probe.

Antibody probes are prepared by immunizing suitable mammalian hosts in appropriate immunization protocols using the peptides. Peptides or proteins comprising the NBD are of sufficient length, or if desired, as required to enhance immunogenicity, conjugated to suitable carriers. Methods for preparing immunogenic conjugates with carriers such as BSA, KLH, or other carrier proteins are well known in the art. In some circumstances, direct conjugation using, for

example, carbodiimide reagents may be effective; in other instances linking reagents such as those supplied by Pierce Chemical Co. may be desirable to provide accessibility to the hapten. The hapten peptides can be extended at either the amino or carboxy terminus with a cysteine residue or interspersed with cysteine residues, for example, to facilitate linking to a carrier. Administration of the immunogens is conducted generally by injection over a suitable time period and with use of suitable adjuvants, as is generally understood in the art. During the immunization schedule, titers of antibodies are taken to determine adequacy of antibody formation.

While the polyclonal antisera produced in this way may be satisfactory for some applications, for pharmaceutical compositions, use of monoclonal preparations is preferred. Immortalized cell lines which secrete the desired monoclonal antibodies may be prepared using the standard method of Kohler & Milstein, (1992) Biotechnology 24, 524–526 or modifications which effect immortalization of lymphocytes or spleen cells, as is generally known. The immortalized cell lines secreting the desired antibodies are screened by immunoassay in which the antigen is the peptide hapten, peptide or protein. When the appropriate immortalized cell culture secreting the desired antibody is identified, the cells can be cultured either in vitro or by production in ascites fluid.

The desired monoclonal antibodies may be recovered from the culture supernatant or from the ascites supernatant. Fragments of the monoclonals or the polyclonal antisera which contain the immunologically significant portion can be used as antagonists, as well as the intact antibodies. Use of immunologically reactive fragments, such as the Fab, Fab' of $F(ab')_2$ fragments is often preferable, especially in a therapeutic context, as these fragments are generally less immunogenic than the whole immunoglobulin.

The antibodies or fragments may also be produced, using current technology, by recombinant means. Antibody regions that bind specifically to the desired regions of the protein can also be produced in the context of chimeras with multiple species origin.

Agents that are assayed in the above method can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a non-random basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that comprises the NBD on IKKβ or the IKKβ binding domain on NEMO. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to the amino acid sequence of SEQ ID NO: 2 or a peptide with conservative substitutions thereof.

The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention.

The peptide agents of the invention can be prepared using standard solid phase (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

The present invention further provides isolated nucleic acid molecules that encode the peptide having the NBD and conservative nucleotide substitutions thercof, preferably in isolated form. Conservative nucleotide substitutions include nucleotide substitutious which do not effect the coding for a particular amino acid as most amino acids have more than one codon (see King & Stansfield (Editors), A Dictionary of Genetics, Oxford University Press, 1997 at page 19). Conservative nucleotide substitutions therefore also include silent mutations and differential codon usage. For example, the invention includes the nucleic acid (SEQ ID NO: 1) encoding the peptide set forth in SEQ ID NO: 2, and conservative nucleotide substitutions thereof. The invention also includes nucleic acids encoding the peptides set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 and conservative nucleotide substitutions thereof. Any nucleic acid that encodes the peptides set forth in SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 is encompassed in the invention, given the multiple permutations of nucleotide sequences possible which encode these peptides.

Specific examples of nucleic acids encompassed by this invention include, but are not limited to the following: (1) the amino acids of the peptide of SEQ ID NO: 2 can be encoded by the nucleic acid sequence TTAGATTGGTCT-FGGTTA (SEQ ID NO: 24) or TTGGACTGGTCCTG-GCTA (SEQ ID NO: 25); and (2) the amino acids of the peptide of SEQ ID NO: 15 can be encoded by the nucleic acid sequence TTAGATTGGTCTTATCTG (SEQ ID NO: 26) or CTTGACTGGTCATACTRA (SEQ ID NO: 27).

As used herein, a nucleic acid molecule is said to be "isolated" when the nucleic acid molecule is substantially separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid. Modifications to the primary structure of the nucleic acid itself by deletion, addition, or alteration of the amino acids incorporated into the protein sequence during translation can be made without destroying the activity of the peptide. Such substitutions or other alterations result in peptide having an amino acid sequence encoded by a nucleic acid falling within the contemplated scope of the present invention.

Another class of agents of the present invention are antibodies immunoreactive with critical positions of proteins of the invention. Antibody agents are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic regions, those portions of the protein intended to be targeted by the antibodies.

C. High Throughput Assays

Introduction—The power of high throughput screening is utilized to the search for new compounds which are capable of interacting with the NEMO binding domain. For general information on high-throughput screening, see, for example, Cost-Effective Strategies for Automated and Accelerated High-Throughput Screening, IBCS Biomedical Library Series, IBC United States Conferences, 1996; Devlin (Editor), High Throughput Screening, Marcel Dekker 1998; U.S. Pat. No. 5,763,263. High throughput assays utilize one or more different assay techniques.

1. Immunodiagnostics and Immunoassays—These are a group of techniques used for the measurement of specific

biochemical substances, commonly at low concentrations in complex mixtures such as biological fluids, that depend upon the specificity and high affinity shown by suitably prepared and selected antibodies for their complementary antigens. A substance to be measured must, of necessity, be antigenic—either an immunogenic macromolecule or a haptenic small molecule. To each sample a known, limited amount of specific antibody is added and the fraction of the antigen combining with it, often expressed as the bound:free ratio, is estimated, using as indicator a form of the antigen labeled with radioisotope (radioimmunoassay), fluorescent molecule (fluoroimmunoassay), stable free radical (spin immunoassay), enzyme (enzyme immunoassay), or other readily distinguishable label.

Antibodies can be labeled in various ways, including: enzyme-linked immunosorbent assay (ELISA); radioimmuno assay (RIA); fluorescent immunoassay (FIA); chemiluminescent immunoassay (CLIA); and labeling the antibody with colloidal gold particles (immunogold).

Common assay formats include the sandwhich assay, competitive or competition assay, latex agglutination assay, homogeneous assay, microtitre plate format and the microparticle-based assay.

Enzyme-linked immunosorbent assay (ELISA)—ELISA is an immunochemical technique that avoids the hazards of radiochemicals and the expense of fluorescence detection systems. Instead, the assay uses enzymes as indicators. ELISA is a form of quantitative immunoassay based on the use of antibodies (or antigens) that are linked to an insoluble carrier surface, which is then used to "capture" the relevant antigen (or antibody) in the test solution. The antigen-antibody complex is then detected by measuring the activity of an appropriate enzyme that had previously been covalently attached to the antigen (or antibody).

For information on ELISA techniques, see, for example, Crowther, ELISA: Theory and Practice (Methods in Molecular Biology, Vol. 42), Humana Press, 1995; Challacombe & Kemeny, ELISA and Other Solid Phase Immunoassays: Theoretical and Practical Aspects, John Wiley, 1998; Kemeny, A Practical Guide to ELISA, Pergamon Press, 1991; Ishikawa, Ultrasensitive and Rapid Enzyme Immunoassay (Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 27), Elsevier, 1991.

Colorimetric Assays for Enzymes—Colorimetry is any method of quantitative chemical analysis in which the concentration or amount of a compound is determined by comparing the color produced by the reaction of a reagent with both standard and test amounts of the compound, often using a calorimeter. A colorimeter is a device for measuring color intensity or differences in color intensity, either visually or photoelectrically.

Standard calorimetric assays of beta-galactosidase enzymatic activity are well known to those skilled in the art (see, for example, Norton et al., (1985) Mol. Cell. Biol. 5, 281–290). A colorimetric assay can be performed on whole cell lysates using O-nitrophenyl-beta-D-galactopyranoside (ONPG, Sigma) as the substrate in a standard calorimetric beta-galactosidase assay (Sambrook et al.,(1989) Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Press). Automated colorimetric assays are also available for the detection of beta-galactosidase activity, as described in U.S. Pat. No. 5,733,720.

Immunofluorescence Assays—Immunofluorescence or immunofluorescence microscopy is a technique in which an antigen or antibody is made fluorescent by conjugation to a fluorescent dye and then allowed to react with the complementary antibody or antigen in a tissue section or smear. The location of the antigen or antibody can then be determined by observing the fluorescence by microscopy under ultraviolet light.

For general information on immunofluorescent techniques, see, for example, Knapp et al., (1978) Immunofluorescence and Related Staining Techniques, Elsevier; Allan, (1999) Protein Localization by Fluorescent Microscopy: A Practical Approach (The Practical Approach Series, Vol. 218) Oxford University Press; Beutner, (1983) Defined Immunofluorescence and Related Cytochemical Methods, New York Academy of Sciences; Caul, (1993). Immunofluorescence Antigen Detection Techniques in Diagnostic Microbiology, Cambridge University Press. For detailed explanations of immunofluorescent techniques applicable to the present invention, see, U.S. Pat. Nos. 5, 912, 176; 5, 869, 264; 5, 866, 319; and 5, 861 259.

Biochips—The peptides of the invention can be used on an array or microarray for high-throughput screening for agents which interact with either the nucleic acids of the invention or their corresponding proteins.

An "array" or "microarray" generally refers to a grid system which has each position or probe cell occupied by a defined nucleic acid fragments also known as oligonucleotides. The arrays themselves are sometimes referred to as "chips" or "biochips" which are high-density nucleic acid and peptide microarrays often having thousands of probe cells in a variety of grid styles.

A typical molecular detection chip includes a substrate on which an array of recognition sites, binding sites or hybridization sites are arranged. Each site has a respective molecular receptor which binds or hybridizes with a molecule having a predetermined structure. The solid support substrates which can be used to form surface of the array or chip include organic and inorganic substrates, such as glass, polystyrene, polyimides, silicon dioxide and silicon nitride. For direct attachment of probes to the electrodes, the electrode surface must be fabricated with materials capable of forming conjugates with the probes.

Once the array is fabricated, a sample solution is applied to the molecular detection chip and molecules in the sample bind or hybridize at one or more sites. The sites at which binding occurs are detected, and one or more molecular structures within the sample are subsequently deduced. Detection of labeled batches is a traditional detection strategy and includes radioisotope, fluorescent and biotin labels, but other options are available, including electronic signal transduction.

The methods of this inventiou will find particular use wherever high through-put of samples is required. In particular, this invention is useful in ligand screening settings and for determining the composition of complex mixtures.

Polypeptides are an exemplary system for exploring the relationship between structure and function in biology. When the twenty naturally occurring amino acids are condensed into a polymeric molecule they form a wide variety of three-dimensional configurations, each resulting from a particular amino acid sequence and solvent condition. For example, the number of possible polypeptide configurations using the twenty naturally occurring amino acids for a polymer five amino acids long is over three million. Typical proteins are more than one-hundred amino acids in length.

In typical applications, a complex solution containing one or more substances to be characterized contacts a polymer array comprising polypeptides. The polypeptides of the invention can be prepared by classical methods known in the art, for example, by using standard solid phase techniques.

The standard methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis and recombinant DNA technology (see Merrifield, (1963) Am. Chem. Soc. 85, 2149–2152).

In a preferred embodiment, the polypeptides or proteins of the array can bind to other co-receptors to form a heteroduplex on the array. In yet another embodiment, the polypeptides or proteins of the array can bind to peptides or small molecules.

D. Uses for Agents That Interact at the NBD

As provided in the Examples, agents that modulate or up- or down-regulate the expression of NEMO or agents such as agonists or antagonists of at least one activity of NEMO may be used to modulate biological and pathologic processes associated with the NEMO or IKKβ function and activity. In particular, these agents effect NF-κB mediated processes by interacting with NEMO and can be used to modulate biological or pathological processes associated with NF-κB.

As used herein, a subject can be any mammal, so long as the mammal is in need of modulation of a pathological or biological process mediated by a protein of the invention. The term "mammal" is meant to identify an individual belonging to the class Mammalia. The invention is particularly useful in the treatment of human subjects.

Pathological processes refer to a category of biological processes which produce a deleterious effect. For example, unregulated expression of NF-κB is associated with pro-inflammatory processes underlying certain pathological processes. As used herein, an agent is said to modulate a pathological process when the agent reduces the degree or severity of the process. For instance, pro-inflammatory responses may be prevented or pathological processes modulated by the administration of agents which reduce, promote or modulate in some way the expression or at least one activity of a NEMO or IKKβ. Agents can therefore be used to treat diseases with an NF-κB inflammatory component, such disease include but are not limited to; osteoporosis, rheumatoid arthritis, atherosclerosis, asthma (Ray & Colin, (1999) J. Clin. Invest. 104, 985–993; Christman et al., (2000) Chest 117, 1482–1487) and Alzheimer's disease. For a review of diseases with an NF-κB inflammatory component, see Epstein, (1997) New Eng. J. Med. 336, 1066–1071; Lee et al., (1998) J. Clin. Pharmacol. 38, 981–993; Brand et al., (1 997) Exp. Physiol. 82, 297–304.

Pathological processes associated with a pro-inflammatory response in which the agents of the invention would be useful for treatment include, but are not limited to, asthma, allergies such as allergic rhinitis, uticaria, anaphylaxis, drug sensitivity, food sensitivity, etc. and the like; cutaneous inflammation such as dermatitis, eczema, psoraisis, contact dermatitis, sunburn, aging, etc. and the like; arthritis such as osteoarthritis, psoriatic arthritis, lupus, spondylarthritis, etc. and the like. These agents also are useful for treating chronic obstruction pulmonary disease and chronic inflammatory bowel disease. The peptides of the present invention can be used to replace corticosteroids in any application in which corticosteroids are used including immunosuppression in transplants and cancer therapy.

The agents of the present invention can be provided alone, or in combination with other agents that modulate a particular pathological process. For example, an agent of the present invention can be administered in combination with anti-inflammatory agents. As used herein, two agents are said to be administered in combination when the two agents are administered simultaneously or are administered independently in a fashion such that the agents will act at the same time.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. Alternatively, or concurrently, administration may be by the oral route. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

E. Pharmaceutical Preparations

The invention also includes pharmaceutical compositions comprising the agents of the invention together with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Gennaro et al., (1995) Remington's Pharmaceutical Sciences, Mack Publishing Company. In addition to the pharmacologically active agent, the compositions of the present invention may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically for delivery to the site of action. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and dextran. Optionally, the suspension may also contain stabilizers. Liposomes can also be used to encapsulate the agent for delivery into the cell.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for enteral, parenteral or topical administration. Indeed, all three types of formulations may be used simultaneously to achieve systemic administration of the active ingredient.

Suitable formulations for oral administration include hard or soft gelatin capsules, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

The agents of the present invention can be administered via parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal or buccal routes. Alternatively, or concurrently, administration may be by the oral route or by inhalation or lavage, directly to the lungs The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The agents used in the method of treatment of this invention may be administered systemically or topically, depending on such considerations as the condition to be treated, need for site-specific treatment, quantity of drug to be administered and similar considerations.

Topical administration may be used. Any common topical formation such as a solution, suspension, gel, ointment or salve and the like may be employed. Preparation of such topical formulations are well described in the art of phar-

maceutical formulations as exemplified, for example, by Remington's Pharmaceutical Sciences. For topical application, these compounds could also be administered as a powder or spray, particularly in aerosol form. The active ingredient may be administered in pharmaceutical compositions adapted for systemic administration. As is known, if a drug is to be administered systemically, it may be confected as a powder, pill, tablet or the like or as a syrup or elixir for oral administration. For intravenous, intraperitoneal or intra-lesional administration, the compound will be prepared as a solution or suspension capable of being administered by injection. In certain cases, it may be useful to formulate these compounds in suppository form or as an extended release formulation for deposit under the skin or intramuscular injection. In a preferred embodiment, the compounds of this invention may be administered by inhalation. For inhalation therapy the compound may be in a solution useful for administration by metered dose inhalers or in a form suitable for a dry powder inhaler.

An effective amount is that amount which will modulate the activity or alter the level of a target protein. A given effective amount will vary from condition to condition and in certain instances may vary with the severity of the condition being treated and the patient's susceptibility to treatment. Accordingly, a given effective amount will be best determined at the time and place through routine experimentation. However, it is anticipated that in the treatment of a tumor in accordance with the present invention, a formulation containing between 0.001 and 5 percent by weight, preferably about 0.01 to 1 percent, will usually constitute a therapeutically effective amount. When administered systemically, an amount between 0.01 and 100 mg per kg body weight per day, but preferably about 0.1 to 10 mg per kg, will effect a therapeutic result in most instances.

In practicing the methods of this invention, the compounds of this invention may be used alone or in combination, or in combination with other therapeutic or diagnostic agents. In certain preferred embodiments, the compounds of this invention may be coadministered along with other compounds typically prescribed for these conditions according to generally accepted medical practice. The compounds of this invention can be utilized in vivo, ordinarily in mammals, preferably in humans.

In still another embodiment, the compounds of the invention may be coupled to chemical moieties, including proteins that alter the functions or regulation of target proteins for therapeutic benefit. These proteins may include in combination other inhibitors of cytokines and growth factors that may offer additional therapeutic benefit in the treatment of disorders associated with inflammation. In addition, the molecules of the invention may also be conjugated through phosphorylation to biotinylate, thioate, acetylate, iodinate using any of the cross-linking reagents well known in the art.

F. Molecular Biology, Microbiology and Recombinant DNA Technique

In accordance with the present invention, as described above or as discussed in the Examples below, there may be employed conventional molecular biology, microbiology and recombinant DNA techniques. Such techniques are explained fully in the literature. See for example, Sambrook et al., (1989) Molecular Cloning—A Laboratory Manual, Cold Spring Harbor Press; Glover, (1985) DNA Cloning: A Practical Approach; Gait, (1984) Oligonucleotide Synthesis; Harlow & Lane, (1988) Antibodies—A Laboratory Manual, Cold Spring Harbor Press; Roe et al., (1996) DNA Isolation and Sequencing: Essential Techniques, John Wiley; and Ausubel et. al., (1995) Current Protocols in Molecular Biology, Greene Publishing.

G. Antisense RNA

Antisense molecules are RNA or single-stranded DNA molecules with nucleotide sequences complementary to a specified mRNA. When a laboratory-prepared antisense molecule is injected into cells containing the normal mRNA transcribed by a gene under study, the antisense molecule can base-pair with the mRNA, preventing translation of the mRNA into protein. The resulting double-stranded RNA or RNA/DNA is digested by enzymes that specifically attach to such molecules. Therefore, a depletion of the mRNA occurs, blocking the translation of the gene product so that antisense molecules find uses in medicine to block the production of deleterious proteins. Methods of producing and utilizing antisense RNA are well known to those of ordinary skill in the art (see, for example, Lichtenstein & Nellen (Editors), Antisense Technology: A Practical Approach, Oxford University Press, 1997; Agrawal & Crooke, Antisense Research and Application (Handbook of Experimental Pharmacology, Vol. 131), Springer Verlag, 1998; Gibson, Antisense and Ribozyme Methodology: Laboratory Companion, Chapman & Hall, 1997; Mol & Van Der Krol, Antisense Nucleic Acids and Proteins, Marcel Dekker; Weiss, Antisense Oligodeoxynucleotides and Antisense RNA: Novel Pharmacological and Therapeutic Agents, CRC Press, 1997; Stanley et al., (1993) Antisense Research and Applications, CRC Press; Stein & Krieg, (1998) Applied Antisense Oligonucleotide Technology).

Antisense molecules and ribozymes of the invention may be prepared by any method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

RNA molecules may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2'O-methyl rather than phosphodiesterase linkages within the backbone of the molecule. This concept can be extended by the inclusion of nontraditional bases such as inosine, queosine, and wybutosine, as well as acetyl-, methyl-, thio-, and similarly modified forms of adenine, cytidine, guanine, thymine, and uridine which are not as easily recognized by endogenous endonucleases.

H. Fusion Proteins

A fusion protein is an expression product resulting from the fusion of two genes. Such a protein may be produced, e.g., in recombinant DNA expression studies or, naturally, in certain viral oncogenes in which the oncogene is fused to gag.

The production of a fusion protein sometimes results from the need to place a cloned eukaryotic gene under the control of a bacterial promoter for expression in a bacterial system. Sequences of the bacterial system are then frequently expressed linked to the eukaryotic protein. Fusion proteins are used for the analysis of structure, purification, function, and expression of heterologous gene products.

A fused protein is a hybrid protein molecule which can be produced when a nucleic acid of interest is inserted by recombinant DNA techniques into a recipient plasmid and displaces the stop codon for a plasmid gene. The fused

protein begins at the amino end with a portion of the plasmid protein sequence and ends with the protein of interest.

The production of fusion proteins is well known to one skilled in the art (See, e.g., U.S. Pat. Nos. 5, 908, 756; 5, 907, 085; 5, 906, 819; 5, 905, 146; 5, 895, 813; 5, 891, 643; 5, 891, 628; 5, 891, 432; 5, 889, 109; 5, 889, 158; 5, 888, 981; 5, 888, 773; 5, 886, 150; 5, 886, 149; 5, 885, 833; 5, 885, 803; 5, 885, 779; 5, 885, 580; 5, 883, 124; 5, 882, 941; 5, 882, 894; 5, 882, 864; 5, 879, 917; 5, 879, 893; 5, 876, 972; 5, 874, 304; and 5, 874, 290). For a general review of the construction, properties, applications and problems associated with specific types of fusion molecules used in clinical and research medicine, see, e.g., Chamow et al., (1999) Antibody Fusion Proteins, John Wiley.

I. Peptide Mimetics.

This invention also includes peptide mimetics which mimic the three-dimensional structure of the NBD on IKKβ and block NEMO binding at the NBD by binding to NEMO. Such peptide mimetics may have significant advantages over naturally-occurring peptides, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others.

In one form, mimetics are peptide-containing molecules that mimic elements of protein secondary structure. See, for example, Johnson et al., (1993) Peptide Turn Mimetics in Biotechnology and Pharmacy, Pezzuto et al., (Editors) Chapman & Hall. The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and antigen. A peptide mimetic is expected to permit molecular interactions similar to the natural molecule.

In another form, peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compounds are also referred to as "peptide mimetics" or "peptidomimetics" (Fauchere, (1986) Adv. Drug Res. 15, 29–69; Veber & Freidinger, (1985) Trends Neurosci. 8, 392–396; and Evans et al., (1987) J. Med. Chem. 30, 1229–1239, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling.

Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptide mimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biochemical property or pharmacological activity), such as the NBD, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: —CH_2NH—, —CH_2S—, —CH_2—CH_2—, —CH=CH— (cis and trans), —$COCH_2$—, —CH(OH)CH_2—, and —CH_2SO—, by methods known in the art and further described in the following references: Weinstein, (1983) Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Marcel Dekker; Morley, (1980) Trends Pharmacol. Sci. 1, 463–468 (general review); Hudson et al., (1979) Int. J. Pept. Protein Res. 14, 177–185 (—CH_2NH—, CH_2CH_2—); Spatola et al., (1986) Life Sci. 38, 1243–1249 (—CH_2—S); Hann, (1982) J. Chem. Soc. Perkin Trans. 1, 307–314 (—CH—CH—, cis and trans); Almquist et al., (1980) J. Med. Chem. 23, 1392–1398 (—$COCH_2$—); Jennings-White et al., (1982) Tetrahedron Lett. 23, 2533 (—$COCH_2$—); U.S. patent application Ser. No. 4, 424, 207 (—CH(OH)CH_2—); Holladay et al., (1983) Tetrahedron Lett. 24, 4401–4404 (—C(OH)CH_2—); and Hruby, (1982) Life Sci. 31, 189–199 (—CH_2—S—), each of which is incorporated herein by reference.

Labeling of peptide mimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptide mimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (e.g., are not contact points in NBD-NEMO complexes) to which the peptide mimetic binds to produce the therapeutic effect. Derivitization (e.g., labeling) of peptide mimetics should not substantially interfere with the desired biological or pharmacological activity of the peptide mimetic.

NBD peptide mimetics can be constructed by structure-based drug design through replacement of amino acids by organic moieties (see, for example, Hughes, (1980) Philos. Trans. R. Soc. Lond. 290, 387–394; Hodgson, (1991) Biotechnol. 9, 19–21; Suckling, (1991) Sci. Prog. 75, 323–359).

The use of peptide mimetics can be enhanced through the use of combinatorial chemistry to create drug libraries. The design of peptide mimetics can be aided by identifying amino acid mutations that increase or decrease binding of NEMO at the NBD on IKKβ. For example, such mutations as identified in Table 1. Approaches that can be used include the yeast two hybrid method (see Chien et al., (1991) Proc. Natl. Acad. Sci. USA 88, 9578–9582) and using the phage display method. The two hybrid method detects protein-protein interactions in yeast (Fields et al., (1989) Nature 340, 245–246). The phage display method detects the interaction between an immobilized protein and a protein that is expressed on the surface of phages such as lambda and M13 (Amberg et al., (1993) Strategies 6, 2–4; Hogrefe et al., (1993) Gene 128, 119–126). These methods allow positive and negative selection for protein-protein interactions and the identification of the sequences that determine these interactions.

For general information on peptide synthesis and peptide mimetics, see, for example, Jones, (1992) Amino Acid and Peptide Synthesis, Oxford University Press; Jung, (1997) Combinatorial Peptide and Nonpeptide Libraries: A Handbook, John Wiley; and Bodanszky et al., (1993). Peptide Chemistry: A Practical Textbook, 2nd Revised Edition, Springer Verlag each of which is hereby incorporated in its entirety.

J. Transgenic Animals

Transgenic animals are genetically modified animals into which recombinant, exogenous or cloned genetic material has been experimentally transferred. Such genetic material is often referred to as a transgene. The nucleic acid sequence of the transgene may be integrated either at a locus of a genome where that particular nucleic acid sequence is not otherwise normally found or at the normal locus for the transgene. The transgene may consist of nucleic acid sequences derived from the genome of the same species or of a different species than the species of the target animal.

The term "germ cell line transgenic animal" refers to a transgenic animal in which the genetic alteration or genetic information was introduced into a germ line cell, thereby conferring the ability of the transgenic animal to transfer the genetic information to offspring. If such offspring in fact possess some or all of that alteration or genetic information, then they too are transgenic animals.

The alteration or genetic information may be foreign to the species of animal to which the recipient belongs, foreign only to the particular individual recipient, or may be genetic

information already possessed by the recipient. In the last case, the altered or introduced gene may be expressed differently than the native gene.

Transgenic animals can be produced by a variety of different methods including transfection, electroporation, microinjection, gene targeting in embryonic stem cells and recombinant viral and retroviral infection (see, e.g., U.S. Pat. No. 4,736,866; U.S. Pat. No. 5,602,307; Mullins et al., (1993) Hypertension 22, 630–633; Brenin et al., (1997) Surg. Oncol. 6, 99–110; Tuan, (1997) Recombinant Gene Expression Protocols, Methods in Molecular Biology No. 62, Humana Press).

A number of recombinant or transgenic mice have been produced, including those which express an activated oncogene sequence (U.S. Pat. No. 4,736,866); express simian SV40 T-antigen (U.S. Pat. No. 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-1) (U.S. Pat. No. 5,731,490); exhibit dopaminergic dysfunction (U.S. Pat. No. 5,723,719); express at least one human gene which participates in blood pressure control (U.S. Pat. No. 5,731,489); display greater similarity to the conditions existing in naturally occurring Alzheimer's disease (U.S. Pat. No. 5,720, 936); have a reduced capacity to mediate cellular adhesion (U.S. Pat. No. 5,602,307); possess a bovine growth hormone gene (Clutter et al., (1996) Genetics 143, 1753–1760) or, are capable of generating a fully human antibody response (Zou et al., (1993) Science 262, 1271–1274).

While mice and rats remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine animals, including sheep, goats, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs. (see Kim et al., (1997) Mol. Reprod. Dev. 46, 515–526; Houdebine, (1995) Reprod. Nutr. Dev. 35, 609–617; Petters, (1994) Reprod. Fertil. Dev. 6, 643–645; Schnieke et al., (1997) Science 278, 2130–2133; Amoah, (1997) J. Animal Science 75, 578–585).

The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method which favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals are readily available to one skilled in the art, including the disclosures in U.S. Pat. No. 5,489,743 and U.S. Pat. No. 5,602,307.

The present invention comprises transgenic animals expressing a gene encoding the NBD, and mutations of that gene resulting in conservative and non-conservative amino acid substitutions when compared to the wild type gene.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1

Identification of NEMO Binding Domain on IKKβ

To identify the NEMO-interacting domain of IKKβ we performed in vitro pull down assays (Zhong et al., (1997) Cell 89, 413–424) using a bacterially expressed version of full length NEMO fused at its NH_2-terminus to glutathione S-transferase (GST-NEMO; FIG. 1A). Various truncation mutants lacking different functional domains of IKKβ (catalytic domain, leucine zipper and helix-loop-helix; FIG. 1A) were constructed.

All sub-cloning and mutagenesis of full length cDNA clones of IKKα and IKKβ was performed by PCR using cloned Pfu DNA-polymerase (Stratagene). The wild-type and mutated IKKβ cDNA were inserted into the KpnI and NotI restriction sites of pcDNA-3 or pcDNA-3.1-xpress (Invitrogen) and all IKKα cDNAs were inserted into the EcoRI and XhoI sites of the same vectors. FLAG-tagged versions of both kinases were constructed by subcloning into pFLAG-CMV-2 (Sigma). For GST-IKKβ-(644–756), the PCR fragment was inserted into the EcoRI and XhoI sites of pGEX-4T1 (Pharmacia). Full length cDNA encoding human NEMO was obtained by reverse transcriptase (RT)-PCR from HeLa cell mRNA using the Expand™ Long Template PCR System (Boehringer Mannheim) and the primer pair (5'-ATAGACGAATTCAATAGGCACCTCTGGAAG) (SEQ ID NO: 20) and (3'-TAGGACCTCGAGCTACTCAATGCACTCCATG) (SEQ ID NO: 21). The resulting PCR fragment was inserted into the EcoRI and XhoI sites of pcDNA-3 or pcDNA-s.1-xpress. All subsequent NEMO mutants were constructed by PCR using Pfu DNA-polymerase. GST-NEMO was constructed by sub-cloning the full-length cDNA into the EcoRI and XhoI sites of pGEX-4T1.

These mutants were labeled by in vitro translation with [^{35}S]-methionine (input; FIG. 1B), mixed with either GST alone or GST-NEMO, and precipitated using glutathione-agarose. None of the mutants interacted with GST alone, whereas wild-type and all three NH_2-terminal truncations of IKKβ (307–756, 458–756 and 486–756) interacted with GST-NEMO (FIG. 1B (right panel). In contrast, none of the COOH-terminal truncation mutants (1–456, 1–605 or 1–644) precipitated with GST-NEMO. These results demonstrate that NEMO interacts with a region in the COOH-terminus of IKKβ distal to the helix-loop-helix (HLH) domain. A mutant consisting of only the region from amino acid 644 (immediately after the HLH) to the COOH-terminus (residue 756) of IKKβ was constructed next. As shown in FIG. 1C, this mutant did not precipitate with GST but did interact with GST-NEMO confirming that this region mediates the interaction between these two molecules.

The effects of IKKβ-(644–756) on IL-1β- and TNFα-induced NF-κB activation by transiently transfecting HeLa cells with the mutant together with an NF-κB-dependent reporter plasmid (pBIIX-luciferase) was tested next (Kopp & Ghosh, (1994) Science 265, 956–959). For transfection studies, HeLa and COS cells were seeded into either twenty-four well (1×10^5 cells/well) or six well (5×10^5 cells/well) plates and grown for twenty-four hours before transfection of DNA with Fugene6 (Roche) according to the manufacturer's protocol. Cells in twenty-four well and six well trays received a total of 1 μg or 2 μg of DNA respectively. After forty-eight hours cells were lysed with TNT (200 uM NaCl, 20 mM Tris-pH 8.0, 1% Triton-100) and the lysate were used for either immunoprecipitation or luciferase assay (Primage Luciferase Assay System).

FIG. 1D shows that IKKβ-(644–756) inhibited NF-κB activation induced by these cytokines in a dose-dependent manner. These results indicate that IKKβ-(644–756) acts as a dominant-negative by titrating endogenous NEMO out of the core IκB-kinase complex. Without the recruitment of regulatory proteins by NEMO, IKKβ becomes refractory to IL-1β- and TNFα-induced signals that should otherwise cause its activation.

Structurally, NEMO consists of extensive α-helical regions containing two prominent stretches of coiled-coil and a leucine-zipper motif, and a COOH-terminal zinc-finger domain (FIG. 2A) (Mercurio et al., (1999) Mol. Cell. Biol. 19, 1526–1538; Yamaoka et al., (1998) Cell 93, 1231–1240; Rothwarf et al, (1998) Nature 395, 297–300). Previous studies attempting to identify the region of NEMO required for its interaction with IKKβ have generated conflicting results (Harhaj et al., (1999) J. Biol. Chem. 274, 15297–15300). To address this question GST-pull-down assays using a GST-fusion protein of IKKβ-(644–756) (FIG. 2A) and various [^{35}S]-methionine-labeled truncation mutants of NEMO (FIG. 2A) were performed. FIG. 2A (right panel) summarizes the results of these experiments in which it was demonstrated that IKKβ-(644–756) interacted with NEMO-(1–196), -(1–302) and -(44–419) but not NEMO-(197–419) or -(86–419). Identical results were obtained from immuno-precipitation studies using lysate of COS or HEK293 cells transiently transfected with FLAG-tagged IKKβ and the NEMO mutants (data not shown).

For all immunoprecipitations HeLa or COS cells grown in six well trays were lysed in 500 μl TNT. FLAG-tagged proteins were precipitated from lysate of transfected cells for two hours at 4° C. using 20 μl of anti-FLAG (M2)-conjugated agarose beads (Sigma). Immunoprecipitations of endogenous IKKβ or NEMO were performed using 1 μg of specific rabbit polyclonal antibodies (Santa Cruz) plus 20 μl of Protein-A sepharose (Amersham-Pharmacia). For immunoblotting, precipitates were washed three times with TNT, twice with PBS then suspended in SDS-sample buffer. Proteins were separated by SDS-PAGE (10%), transferred to PVDF membranes and visualized by enhanced chemiluminescence (Amersham-Pharmacia).

These results establish that the interaction domain lies between residues 44 and 86, a region including the first α-helix of NEMO. A mutant was therefore made in which α-helix up to the first coiled-coil domain was deleted (residues 150-L93; delαH). This mutant did not interact with IKKβ-(644–756) (FIG. 2B). Furthermore transfection studies using pBIIX-luciferase demonstrated that delαH inhibited TNFα-induced NF-κB activity (FIG. 2C) confirming previous reports that the COOH-terminus of NEMO which can not interact with IKKβ, is a dominant-negative inhibitor of NF-κB (Mercurio et al., (1999) Mol. Cell. Biol. 19, 1526–1538; Rothwarf et al., (1998) Nature 395, 297–300). Taken together, FIGS. 1 and 2 show that the interaction between IKKβ and NEMO occurs via the COOH-terminus of IKKβ and the first α-helical region of NEMO. These findings suggest a model in which the NH_2-terminus of NEMO anchors it to the IKK-complex leaving the remainder of the molecule containing several protein-protein interaction domains free and accessible for interacting with upstream regulators of IKK function.

Example 2

NEMO Regulation of IKKβ Function Through Interaction at NBD

To fully characterize the NEMO-interaction domain of IKKβ further truncation mutants between residues V644 and S756 (FIG. 3A) were constructed. Immediately after the HLH, the amino acid sequence to the cysteine at position 662 exhibits 72% identity with IKKα (denoted α_1 in FIG. 3A). Following this, the region up to E707 is a serine-rich domain previously reported to be a target for auto-phosphorylation and to function in down-regulating IKKβ activity after stimulation by pro-inflammatory cytokines (Delhase et al., (1999) Science 284, 309–313). The sequence succeeding this contains no serine residues until position 733. Mutants sequentially omitting each of these regions were [^{35}S]-methionine-labeled and used in GST-pull-down assays as described above. FIG. 3A summarizes the results from these experiments demonstrating that none of the IKKβ mutants precipitated with GST-NEMO and indicating that the interaction domain resides in the extreme COOH-terminus between residues F734 and S756.

Comparison of this short segment of IKKβ with the corresponding region of IKKα reveals two striking structural characteristics (FIG. 3B). First, the sequence from F734 to T744 of IKKβ (α_2, in FIG. 3B) is identical to the equivalent sequence in IKKα (737 to L742 of IKKβ and L738 to L743 of IKKα). Second, the sequence of IKKβ extends beyond the COOH-terminal residue of IKKα (E745) for twelve amino acids comprising a highly acidic region in which five of the residues are glutamates (FIG. 3B). The marked similarity between the α_2-region of IKKβ and the extreme COOH-terminus of IKKα together with previous reports that NEMO does not interact with IKKα in vitro (Mercurio et al., (1999) Mol. Cell. Biol 19, 1526–1538; Yamaoka et al., (1998) Cell 93, 1231–1240; Rothwarf et al., (1998) Nature 395, 297–300) led to the hypothesis that the NEMO-interaction domain would be the glutamate-rich portion of IKKβ (E745 to S756). To test this hypothesis, a truncation mutant omitting this region was made (1–744; FIG. 3C) and investigated for its ability to interact with GST-NEMO. The mutant associated with GST-NEMO to an equal extent as wild-type IKKβ (FIG. 3C); these results have been confirmed by co-immunonoprecipitating epitope-tagged NEMO and IKKβ-(1–744) from lysate of transiently transfected COS cells. These findings demonstrate that the NEMO-interaction domain of IKKβ is within the α_2-region of the COOH-terminus.

HeLa cells were transfected for forty-eight hours with 1 μg/well of the indicated FLAG-tagged constructs followed by immunoprecipitation using anti-FLAG. The immunoprecipitates were incubated in kinase buffer containing [^{32}P]-labeled γATP for fifteen minutes at 30° C. then washed with lysis buffer containing 1% Triton-100. Resulting complexes were separated by SDS-PAGE (10%) and visualized by autoradiography. An immunoblot from identical samples demonstrated equivalent amounts of transfected protein in each lane. HeLa cells transfected for forty-eight hours with FLAG-tagged versions of either IKKβ (wild-type) or IKKβ-(1–733) were also either untreated (–) or treated for seven minutes (+) with TNFα (10 ng/ml). Following lysis and immunoprecipitation using anti-FLAG, immune-complex kinase assay was performed. Identical samples were immunoprecipitated and immunoblotted with anti-FLAG.

IKKβ COOH-terminal truncation mutants were next used to test the effects of NEMO association on basal and induced activity of IKKβ. Truncation of IKKβ at V644, eliminating the serine-rich region (see FIG. 3A), resulted in complete loss of basal auto-phosphorylation. In contrast, a mutant containing the serine-rich region (1–733), exhibited significantly higher levels of auto-phosphorylation than wild-type IKKβ. Intriguingly, the level of auto-phosphorylation of IKKβ-(1–744) which contains the NEMO-binding α_2-region, was identical to that observed with the wild-type kinase. To test the effects that these mutations have on basal kinase activity, mutants were transiently transfected into HeLa cells and NF-κB activity determined by luciferase assay as described in Example 1. IKKβ-(1–644) did not induce NF-κB activity whereas IKKβ-(1–733) caused

increased activation compared with wild-type (FIG. 3D). Furthermore, NF-κB activity induced by IKKβ-(1–744) was identical to that induced by wild-type IKKβ. These results demonstrate that basal auto-phosphorylation and kinase activity of IKKβ is dependent on the ability of NEMO to associate with the kinase. One explanation for these observations may be that NEMO recruits a phosphatase to the IKK-complex that regulates basal IKKβ function by targeting the serine-rich region of the COOH-terminus. Inability to bind NEMO therefore prevents phosphatase recruitment and causes increased phosphorylation within this region.

To directly test the effect that loss of the α_2-region has on the catalytic activity of IKKβ, an immune-complex kinase assay was performed on lysate from transfected HeLa cells. For immune-complex kinases assays, precipitates were washed with TNT then with kinase buffer (20 mM HEPES pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA, 2 mM NaF, 2 mM β-glycerophosphate, 1 mM DTT, 10 μM ATP). Precipitates were then incubated for fifteen minutes at 30° C. in 20 μl of kinase buffer containing GST-IκBα-(1–90) and 10 μCi [^{32}P]-γ-labeled ATP (Amersham-Pharmacia). The substrate was precipitated using glutathione-agarose (Amersham-Pharmacia) and separated by SDS-PAGE (10%). Kinase activity was determined by autoradiography. Phosphorylated proteins associated with the kinase complex appeared on autoradiographs because the immuno-precipitated complex was not removed prior to GST-substrate precipitation.

Activity of IKKβ (wild-type) was low in untreated cells but was markedly enhanced after treatment with TNFα. Consistent with the data presented in FIG. 3D, basal activity of IKKβ-(1–733) was significantly higher than wild-type, however this activity was not further enhanced by treatment with TNFα. Furthermore, basal and TNFα-induced catalytic activity of IKKβ-(1–744) was identical to the activity of IKKβ (WT). In addition to phosphorylated GST-IκBα, auto-phosphorylated IKKβ proteins were also detected. Following TNFα treatment, IKKβ (WT) and IKKβ-(1–744) became rapidly autophosphorylated whereas the already high basal phosphorylation of IKKβ-(1–733) was only slightly enhanced. A previous study showed that auto-phosphorylation serves to down-regulate TNFα-induced IKKβ activity by causing conformational changes within the protein (Delhase et al., (1999) Science 284, 309–313). Taken together, these findings (FIG. 3D) demonstrate that in the absence of NEMO, IKKβ becomes auto-phoshorylated, basally active and refractory to TNFα-induced signals indicating that NEMO plays a fundamental role in the down-regulation as well as activation of IKKβ activity.

An additional band representing a phosphorylated protein appeared only in the samples from TNFα-induced IKKβ (WT) and IKKβ-(1–744) transfected cells. The molecular weight of this protein (49 kDa) strongly suggests that it is endogenous NEMO associated with the precipitated complex. This is supported by the absence of the band in either precipitate (+/− TNFα) from IKKβ-(1–733) transfected cells. This protein has been identified as phosphorylated NEMO by dissociating the precipitated complex in SDS and re-immunoprecipitating [^{32}P]-labeled NEMO using specific anti-NEMO antibodies. Induced phosphorylation of NEMO may therefore represent a further level of a regulation of the activity of the IKK complex.

Example 3

Identification of the NBD on IKKα

Since the α_2-region of IKKβ strongly resembles the COOH-terminus of IKKα (FIG. 3B), the ability of IKKα to interact with NEMO was tested. Immunoprecipitations from lysate of COS cells transiently transfected with xpress-tagged NEMO together with FLAG-tagged versions of either IKKα or IKKβ were performed using anti-FLAG as described in Example 1. FIGS. 4A and 4B show that NEMO interacted equally well with both IKKβ and IKKα. It is possible that in this experiment the interaction with IKKα is not direct but due instead to the formation of a complex containing endogenous IKKβ, FLAG-IKKα and xpress-NEMO. A GST-pull-down assays was therefore performed using GST-NEMO and [^{35}S]-methionine-labeled versions of either wild-type IKKα or a truncated IKKα mutant lacking the eight COOH-terminal amino acids (1–737: FIG. 4C). In agreement with the findings presented above (FIG. 4A), but in contrast to previous reports (Mercurio et al., (1999) Mol. Cell. Biol. 19, 1526–1538; Yamaoka et al., (1998) Cell 93, 1231–1240; Rothwarf et al., (1998) Nature 395, 297–300), wild-type IKKα interacted with NEMO in vitro whereas the truncated mutant did not (FIG. 4C). These results not only demonstrate that IKKα interacts with NEMO but also shows that it does so via the COOH-terminal region containing the six amino acids shared between IKKα and the α_2-region of IKKβ (FIG. 3B). Gene-targeting studies have demonstrated profound differences in the activation of IKKα and IKKβ by TNFα (Woronicz et al., (1997) Science 278, 866–869; Zandi et al., (1997) Cell 91, 243–252; Mercurio et al., (1997) Science 278, 860–866; DiDonato et al., (1997) Nature 388, 548–554; Régnier et al., (1997) Cell 90, 373–383).

The present findings indicate that the basis of this difference is not due to differential recruitment of NEMO (Delhase et al., (1999) Science 284, 309–313; Takeda et al., (1999) Science 284, 313–316; Hu et al., (1999) Science 284, 316–20; Li et al., (1999) Science 284, 321–325; Li et al., (1999) J. Exp. Med. 189, 1839–1845; Li et al., (1999) Genes Dev. 13, 1322–1328; Tanaka et al., (1999) Immunity 10, 421–429). Instead the difference most likely lies in the ability of each kinase to integrate NEMO-associated signaling components into an activation response, presumably through differences in the inherent regulatory features of the individual kinases.

Further evidence that this short COOH-terminal sequence constitutes the NEMO-interaction domain of the IKKs was obtained when we tested the ability of the recently described IKK-related kinase IKK_i (Shimada et al., (1999) Int. Immunol. 11, 1357–1362) to interact with NEMO. Sequence comparison with IKKα and IKKβ (Shimada et al., (1999) Int. Immunol. 11, 1357–1362; Woronicz et al., (1997) Science 278, 866–869; Zandi et al., (1997) Cell 91, 243–52; Mercurio et al., (1997) Science 278, 860–866; DiDonato et al., (1997) Nature 388, 548–554; Régnier et al., (1997) Cell 90, 373–383) reveals that IKK_i does not contain the α_2-region in its COOH-terminus (Shimada et al., (1999) Int. Immunol. 11, 1357–1362) and consistent with this being the NEMO binding domain we found that IKK_i does not interact with GST-NEMO in pull down assays (FIG. 4D). This finding indicates that NEMO is not required for the functional activity of IKK_i and this is supported by the inability of IKK_i to respond to signals induced by either TNFα or IL-1β (Shimada et al., (1999) Int. Immunol. 11, 1357–1362).

Example 4

Mutation of Amino Acid Residues in the NBD

Having determined that the α_2-region of IKKβ, and the equivalent six amino acid sequence of IKKα are critical for interaction with NEMO [designated NEMO binding domain (NBD)], a deletion mutant in IKKβ lacking the six amino

acids from L737 to L742 (del.NBD) was constructed. This deletion mutant did not associate with GST-NEMO (FIG. 4E). Examination of predicted structural and biochemical features of the NBD in context with surrounding residues suggests that it constitutes an inflexible hydrophobic "pocket" within a hydrophilic region of the IKKβ COOH-terminus (FIG. 4F). This suggests a model in which the NBD becomes buried within the first α-helical region of bound NEMO (FIG. 2) preventing its exposure to an aqueous environment thereby maintaining a strong inter-molecular interaction. Whether the interaction is indeed a function of this hydrophobicity remains to be determined, however we found that substitution of either W739 or W741 with alanine prevented association of NEMO with IKKβ (FIG. 4G). Therefore each of these hydrophobic tryptophan residues is critical for maintaining a functional NBD. In addition, mutation of D738 to alanine also prevented NEMO interaction indicating that a negatively charged residue at this position is required for NBD function. In contrast to these mutations, substitution of L737, S740 or L742 with alanine did not affect NEMO binding. To test the effects of these alanine substitutions on IKKβ function, HeLa cells were co-transfected with each of the point mutants together with pBIIX-luciferase reporter. Consistent with the observation that the basal activity of IKKβ is increased in the absence of associated NEMO, IKKβ-(1-733) (FIG. 3D), mutants that did not bind NEMO (D738A, W739A and W741A) activated NF-κB to a greater extent than wild-type IKKβ or IKKβ-(1-744) (FIG. 4H). In contrast, mutants containing substitutions that did not disrupt NEMO association (L737A, S740A and L742A) induced NF-κB to the same level as the controls. These results indicate that NEMO plays a critical role in the down-regulation of intrinsic IKKβ activity.

Further mutations in the NBD were analyzed (see Table 1) for their ability to affect NEMO binding to IKKβ using the GST pulldown assay explained in Example 3.

TABLE 1

Characterized NBD mutants and their ability to bind to NEMO.

NBD Mutants[1]	Binds to NEMO	SEQ ID NO:
LDWSWL	yes	2
LDASAL	no	3
ADWSWL	yes	4
LDWSWA	yes	5
ADWSWA	yes	6
LAWSWL	no	7
LFWSWL	yes	8
LNWSWL	yes	9
LDASWL	no	10
LDFSWL	yes	11
LDYSWL	yes	12
LDWSAL	no	13
LDWSFL	no	14
LDWSYL	no	15
LDWAWL	yes	16
LDWEWL	yes	17

[1]The substituted amino acid residue is indicated by bold face.

Example 5

Agents which Interact with NBD to Block NEMO Binding

The relatively small size of the NE3D makes it an attractive target for the development of compounds aimed at disrupting the core IKK complex. The relevance of this approach was investigated by designing cell-permeable peptides spanning the IKKβ NBD and determining their ability to dissociate the IKKβ-NEMO interaction.

The sequences of the two NBD peptides used in this study were [DRQIKIWFQNRRMKWKK]TALDWSWLQTE (wild-type) (SEQ ID NO: 18) and [DRQIKIWFQNRRMKWKK]TALDASALQTE (mutant) (SEQ ID NO: 19; FIG. 5A). The antennapedia homeodomain sequence (Derossi et al., (1994) J. Biol. Chem. 269, 10444–10450; U.S. Pat. No. 5,888,762; U.S. Pat. No. 6,015, 787; U.S. Pat. No. 6,080,724) is bracketed and the positions of the W→A mutations are underlined. Both petides were dissolved in DMSO to a stock concentration of 20 mM. For all experiments DMSO alone controls were no different from no peptide controls.

The wild-type NBD peptide consisted of the region from T735 to E745 of IKKβ fused with a sequence derived from the third helix of the antennapedia homeodomain that has been shown to mediate membrane translocation (Derossi et al., (1994) J. Biol. Chem. 269, 10444–10450). The mutant was identical except that the tryptophan residues (W739 and W741) in the NBD were mutated to alanine. FIG. 5B shows that the NBD (WT) but not the mutant dose-dependently inhibited in vitro pull-down of [^{35}S]-labeled IKKβ by GST-NEMO and [^{35}S]-labeled NEMO by GST-IKKβ-(644–756). To test the ability of the NBD peptides to enter cells and inhibit the IKKβ-NEMO interaction, HeLa cells were incubated with the peptides for different time periods and immunoprecipitated the IKK complex using anti-NEMO. In agreement with the in vitro data (FIG. 5B), wild-type but not mutant disrupted the formation of the endogendus IKK complex (FIG. 5C).

Example 6

Agents which Block NEMO Function

The effects of the NBD peptides on signal-induced activation of NF-κB were investigated next. Analysis using electrophoretic mobility shift assays (EMSA) also demonstrated that only the wild-type NBD peptide inhibited TNFα-induced activation and nuclear translocation of NF-κB (FIG. 5F). Further, after transfecting HeLa cells with the pBIIX-luciferase reporter, cells were preincubated with wild-type or mutant peptides, treated with TNFα and NF-κB activation measured by the luciferase reporter assay. As shown in FIG. 5H (top panel), the wild-type NBD peptide inhibited TNFα-induced NF-κB activation whereas the mutant had no effect. Interestingly, the basal NF-κB activity was enhanced by treatment with the wild-type peptide (FIG. 5H; bottom panel), a finding which concurs with results from previous mutational analysis (FIGS. 3D and 4H). This indicates that removal of NEMO increases the basal, intrinsic activity of IKK, while abolishing its responsiveness to TNFα. Taken together these results demonstrate that delivery of an intact NBD peptide into cells disrupts the IKKβ-NEMO interaction and prevents pro-inflammatory signals from activating NF-κB. In contrast, transduction with a peptide containing mutations at the tryptophan residues that are critical for maintaining the NEMO interaction has no effect.

Example 7

Agents Capable of Down-regulating E-selectin

Many proteins involved in the initiation and maintenance of inflammatory responses require NF-κB activation for induced expression of their genes (Ghosh et al., (1998)

Annu. Rev. Immunol. 16, 225–260; May & Ghosh, (1998) Immunol. Today 19, 80–88). One such protein, E-selectin, is a leukocyte adhesion molecule expressed on the luminal surface of vascular endothelial cells after activation by pro-inflammatory stimuli such as IL-1 or TNFα (Pober et al., (1986) J. Immunol 436, 1680–1687; Bevilacqua et al., (1987) Proc. Natl. Acad. Sci. USA 84, 9238–9242; Collins et al., (1995) FASEB J. 9, 899–909). Expression of E-selectin and other NF-κB-dependent adhesion molecules is crucial for the arrest and recruitment of leukocytes into sites of acute and chronic inflammation. To assess the anti-inflammatory potential of the NBD peptide, primary human umbilical vein endothelial cells (HUVEC) were pretreated with the wild-type and mutant peptides and E-selectin expression induced with TNFα. Consistent with the effects on basal NF-κB activation (FIG. 5I), the wild-type NBD peptide induced low level expression of E-selectin (FIG. 6A). However, after TNFα-treatment the wild-type but not mutant significantly reduced expression of E-selectin (FIG. 6A). Inhibition by wild-type NBD peptide reduced expression to the level induced by the peptide in the absence of TNFα.

The importance of the present invention can be viewed on two levels. First, Applicants have identified the structural requirements for the association of NEMO with the IKKs and found that association with IKKβ is dependent on three amino acids (D738, W739 and W741) within the NBD. Furthermore, NEMO not only functions in the activation of IKKβ but it also has a critical role in suppressing the intrinsic, basal activity of the IKK complex. The second level of importance is the obvious clinical use for drugs targeting the NBD. Applicants have demonstrated that a cell-permeable peptide encompassing the NBD is able to not only inhibit TNFα-induced NF-κB activation but also reduce expression of E-selectin, an NF-κB-dependent target gene, in primary human endothelial cells. The NBD is only six amino acids long, and therefore it is within the ability of one skilled in the art to design peptido-mimetic compounds that disrupt the core IKK complex. Since the effect of disrupting the complex is to increase the basal activity of the IKK, treatment with an NBD-targeting compound can avoid issues of toxicity, e.g., due to hepatocyte apoptosis, that might arise from administering drugs that completely abolish the activity of NF-κB. Hence, identification of the NBD is a means for the development of novel anti-inflammatory drugs that prevent activating signals from reaching the IKK complex, yet maintain a low level of NF-κB activity and avoid potential toxic side-effects.

Example 8

NBD Peptide-mediated Inhibition of Inflammatory Response in vivo

The NBD peptide was tested for its ability to inhibit inflammatory responses in animals using two distinct models of acute inflammation. In the first model, ear edema was induced in mice using phorbol-12-myristate-13-acetate (PMA) and the effects of topical administration of the NBD peptides were measured. Ear edema using PMA was induced in replicate groups of age and sex matched mice as previously described (Chang et al., (1987) Eur. J. Pharmacol. 142, 197–205). Twenty μl of either NBD peptides (200 μg/ear), dexamethasone (40 μg/ear) or vehicle (DMSO:Ethanol; 25:75 v/v) was applied topically to the right ear of mice thirty minutes before and thirty minutes after the application of 20 μl of PMA (5 μg/ear) dissolved in ethanol. Ear swelling was measured six hours after PMA application using a microgauge and expressed as the mean difference in thickness between the treated (right) and untreated (left) ears. Statistical analysis of the data was performed using the students t-test. A value of $p<0.05$ was considered statistically significant.

FIG. 6C shows that the wild type peptide significantly reduced (77±3% inhibition; $p<0.05$) PMA-induced ear thickening to the level observed with dexamethasone (82±9% inhibition; $p<0.05$). In contrast, the effect observed with an equivalent dose of mutant was insignificant ($p=0.09$). Neither peptide had an effect when administered in the absence of PMA (not shown).

In a second model, peritonitis was induced in mice by intraperitoneal (i.p.) injection of zymosan either alone or in combination with dexamethasone or the NBD peptides. For zymosan-induced peritonitis, measurement of peritoneal exudates and inflammatory cell collections from replicate groups of age and sex matched mice (C57Bl/6NCR) were performed as previously described (Getting et al., (1998) Immunology 95, 625–630). Groups of animals were injected concomitantly with one ml zymosan (1 μg/ml) and either dexamethasone (100 mg/ml) or the NBD peptides (200 μg/ml). The concentration of NOX (nitrate plus nitrite) present in the inflammatory exudates was measured using a colorimetric assay kit (Alexis Corporation) according to the manufacturers protocol.

As shown in FIG. 6D zymosan injection caused an accumulation of inflammatory exudate fluids and migration of polymorphonuclear cells (PMN) into the peritoneum of these animals. Treatment of mice with wild type NBD peptide or dexamethasone significantly reduced exudate formation and PMN accumulation whereas the mutant had no effect.

Various in vivo studies have demonstrated a role for NO in exudate formation and leukocyte migration into inflammatory sites (Ialenti et al., (1992) Eur. J. Pharmacol. 211, 177–182; Ialenti et al., (1993) Br. J. Pharmacol. 110, 701–706; Iuvone et al., (1998) Br. J. Pharmacol. 123, 1325–1330). Therefore the effects of the NBD peptides on NOX accumulation in the peritoneal exudates of zymosan-treated mice were investigated. FIG. 6D (lower panel) shows that dexamethasone and wild-type peptide reduced NOX by 86±7% and 66±4% respectively whereas the mutant had no effect. These results are consistent with previous studies demonstrating that reduction of exudate formation and cell accumulation closely correlate with inhibition of NF-κB activation and reduction of NO formation (D'Acquisto et al., (1999) Eur. J. Pharmacol. 369, 223–236; D'Acquisto et al., (1999) Naunyn-Schmeideberg's Arch. Pharmacol. 360, 670–675). Therefore the wild-type NBD peptide is an effective inhibitor of inflammation in experimental animal models.

Example 9

Inhibition of Osteoclast Differentiation by the NBD Peptide

The processes of bone morphogenesis and remodeling require the maintenance of a balance between the synthesis of bone matrix by osteoblasts and the resorbtion of bone by osteoclasts (Suda et al., (1992) Endocr. Rev. 13, 66–80; Suda et al., (1999) Endocr. Rev. 20, 345–357). Bone-resorbing osteoclasts are multinucleated giant cells that differentiate from myeloid precursors and various soluble factors including colony stimulating factor-1 (CSF-1), Interleukin1 (IL-1), Tumor necrosis factor-α (TNF-α), IL-6 and IL-11 (Suda et

al., (1992) Endocr. Rev. 13, 66–80; Suda et al., (1999) Endocr. Rev. 20, 345–357) that affect osteoclast differentiation at distinct stages. One factor that is critical for osteoclastogenesis is the recently described molecule named RANKL (receptor activator of NF-κB ligand) that is also known as ODF (osteoclast differentiation factor), OPGL (osteoprotegerin ligand) and TRANCE (TNF-related activation-induced cytokine) (Kong et al., (1999) Nature, 397, 315–323; Lacey et al., (1998) Cell 93, 165–176; Suda et al., (1999) Endocr. Rev. 20, 345–357; Wong et al., (1999) J. Leukoc. Biol. 65, 715–724; Yasuda et al., (1998) Proc. Natl. Acad. Sci. USA 95, 3597–3602). The receptor for RANKL is a member of the TNF-receptor family named RANK (receptor activator of NF-κB) (Anderson et al., (1997) Nature 390, 175–179; Dougall et al., (1999) Genes Dev. 13, 2412–2424) and binding of RANKL induces NF-κB activation (Anderson et al., (1997) Nature 390, 175–179; Darnay et al., (1998) J. Biol. Chem. 273, 20551–20555; Darnay et al., (1999) J. Biol. Chem. 274, 7724–31; Suda et al., (1999) Endocr. Rev. 20, 345–357; Wong et al., (1998) J. Biol. Chem. 273, 28355–28359). Moreover, osteoclast differentiation is dependent upon NF-κB activation and gene-targeting studies have demonstrated that mature osteoclasts fail to develop in mice lacking the p50 and p52 NF-κB subunits (Franzoso et al., (1997) Genes Dev. 11, 3482–3496).

Osteoporosis is a severely debilitating disease characterized by an extensive loss of bone mass that is mediated by osteoclast-dependent bone resorbtion (Suda et al., (1992) Endocr. Rev. 13, 66–80; Suda et al., (1999) Endocr. Rev. 20, 345–357). It is therefore possible that selective inhibition of NF-κK activation in osteoclast precursor cells would prevent osteoclast differentiation and provide the basis for therapeutically effective drugs for the treatmcnt of osteoporosis. Therefore the effect of the NBD peptides on osteoclast differentiation was tested using a previously described in vitro model (Jimi et al., (1999) Exp. Cell Res. 247, 84–93). Mouse bone marrow cells plated into 48-well tissue culture trays were incubated with human macrophage-colony stimulating factor (M-CSF; 20 ng/ml) and human RANKL (100 ng/ml) for six days in the absence or presence of various concentrations (6.25, 12.5 and 25 mM) of either mutant or wild-type NBD peptide. The cells were then fixed and stained for the osteoclast phenotypic marker tartrate-resistant acid phosphatase (TRAP) and TRAP-positive muti-nucleated cells containing more than three nuclei were counted as osteoclasts. Triplicate samples were counted and results were calculated as means ±SD. As shown in FIG. 7 the wild type but not mutant peptide dose-dependently inhibited osteoclast differentiation.

This data demonstrates that disruption of the core IKK complex by a cell permeable NBD peptide that inhibits NF-κB activation prevents RANKL-induced osteoclast differentiation indicating that drugs specifically targeting the NBD will be effective for the treatment of osteoporosis. As an extension of these in vitro studies, the same peptides can be analyzed for their effects on osteoporosis in vivo. Ovariectomized mice (Charles River Labs) that exhibit severe osteoporosis are treated with the NBD peptides and the effects on bone density over a timecourse of treatment determined.

Example 10

Effect of NBD Peptides on Other NF-κB Mediated Disorders

In addition, it is also possible to examine the effects of the NBD peptides on asthma. NF-κB activation in bronchiolar epithelial cells, T-cells and bronchiolar macrophages has been observed in the airways of asthmatic patients and in animal models of asthma (Ray & Cohn, (1999) J. Clin. Invest. 104, 985–993; Christman et al., (2000) Chest 117, 1482–1487). In addition, many agents that induce asthma cause NF-κB activation and many of the genes that encode proteins involved in asthma (i.e., leukocyte adhesion molecules, various chemokines, inducible nitric oxide synthase) are NF-κB-dependent. An established mouse model of asthma (Kleeberger et al., (1990) Am. J. Physiol. 258, 313–320) can be used to test the effects of aerosol administration of the NBD peptides on progression of these conditions associated with asthma.

In a similar manner, the effects of the NBD peptides on septic shock can also be measured. Septic shock involves the expression of many NF-κB dependent genes (i.e., TNF, IL-1) that are induced by bacterial endotoxins such as lipopolysaccharide (LPS). LPS comprises the major constituents of the cell walls of gram-negative bacteria and is highly immunogenic and stimulates the production of endogenous pyrogens IL-1 and TNF (Sell et al., (1996) Immunology, Immunopathology & Immunity, Appleton & Lange). To test the effects of the NBD peptides on septic shock, mice are injected with the NBD peptides and-LPS and the survival of animals assessed.

Although the present invention has been described in detail with reference to examples above, it is understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims. All cited patents and publications referred to in this application are herein incorporated by reference in their entirety. The results of the experiments disclosed herein are being submitted to the journal Science, the manuscript of which is herein incorporated by reference in its entirety.

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What is claimed is:

1. An isolated peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17, 18 and 19, wherein said peptide is less than one-hundred amino acids in length.

2. An isolated peptide consisting of an amino acid sequence selected from the group consisting of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19.

3. An isolated peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16and 17, 18 and 19.

4. An isolated peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 16 and 17, 18 and 19, wherein said peptide is a human peptide.

5. A composition comprising the peptide of claim **1**, **2**, **3**, or **4**.

6. The composition of claim **5** further comprising a carrier.

7. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:4.

8. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:5.

9. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:6.

10. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:7.

11. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:8.

12. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:9.

13. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:10.

14. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:11.

15. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:12.

16. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:13.

17. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:14.

18. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:15.

19. The isolated peptide of claim **3**, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:16.

20. The isolated peptide of claim 3, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:17.

21. The isolated peptide of claim 3, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:18.

22. The isolated peptide of claim 3, wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:19.

* * * * *

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Biochemistry

Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1

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Communicated by Leonard A. Herzenberg, Stanford University School of Medicine, Stanford, CA, December 31, 1997 (received for review December 17, 1997)

ABSTRACT **NF-κB is activated by various stimuli including inflammatory cytokines and stresses. A key step in the activation of NF-κB is the phosphorylation of its inhibitors, IκBs, by an IκB kinase (IKK) complex. Recently, two closely related kinases, designated IKKα and IKKβ, have been identified to be the components of the IKK complex that phosphorylate critical serine residues of IκBs for degradation. A previously identified NF-κB-inducing kinase (NIK), which mediates NF-κB activation by TNFα and IL-1, has been demonstrated to activate IKKα. Previous studies showed that mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1), which constitutes the c-Jun N-terminal kinase/stress-activated protein kinase pathway, also activates NF-κB by an undefined mechanism. Here, we show that overexpression of MEKK1 preferentially stimulates the kinase activity of IKKβ, which resulted in phosphorylation of IκBs. Moreover, a catalytically inactive mutant of IKKβ blocked the MEKK1-induced NF-κB activation. By contrast, overexpression of NIK stimulates kinase activities of both IKKα and IKKβ comparably, suggesting a qualitative difference between NIK- and MEKK1-mediated NF-κB activation pathways. Collectively, these results indicate that NIK and MEKK1 independently activate the IKK complex and that the kinase activities of IKKα and IKKβ are differentially regulated by two upstream kinases, NIK and MEKK1, which are responsive to distinct stimuli.**

Exposure of cells to certain cytokines [e.g., tumor necrosis factor (TNF) and interleukin (IL)-1] or environmental stresses (e.g., UV and γ irradiation) leads to activation of the transcription factors NF-κB and c-Jun (1–4). NF-κB is composed of hetero- or homodimers of Rel family proteins and is involved in the inflammatory response, cell adhesion, growth control, and cell death (2, 5, 6). In unstimulated cells, NF-κB is sequestrated in the cytoplasm as a complex with inhibitory proteins called IκBs (1). In the family of IκBs, the most important ones seem to be IκBα, IκBβ, and a recently cloned IκBε (1, 7). Various stimuli to activate NF-κB result in phosphorylation of two serines at the N terminus of IκBα and IκBβ and subsequent degradation of the IκBs, resulting in translocation of NF-κB into the nucleus and activation of target genes. The mutation of the two serine residues Ser-32 and Ser-36 in IκBα decreases phosphorylation and degradation of IκBα protein (8–11). IκBβ and IκBε also have the two conserved serine residues at the N terminus for signal-induced degradation (7, 12, 13). These results indicate that identification of the kinases responsible for the IκB phosphorylation is a critical step for understanding the mechanism of NF-κB activation. A previous study demonstrated that the IκB kinase (IKK) forms a large complex with a molecular mass of 700 kDa, and this complex could be activated by ubiquitination or mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1), a member of the MAP kinase kinase kinase (MAPKKK) family (14). However, the function of MEKK1 in TNF-mediated NF-κB activation still remains controversial (14–18).

The second pathway of stress responses is the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway (3, 4). MEKK1 has been implicated in this pathway, which activates MKK4 that in turn activates JNK/SAPK (19, 20). Then, the JNK/SAPK not only activates c-Jun that constitutes the transcription factor AP-1 but also contributes to apoptosis by an undefined mechanism (21).

Tumor necrosis factor receptor-associated factors (TRAFs) have emerged as signal-transducing molecules through members of the TNF-R superfamily and IL-1R1 (22–35). TRAFs, except for TRAF4, have been shown to directly or indirectly interact with cytoplasmic domains of the TNF-R superfamily molecules and IL-1R1. TRAF2, TRAF5, and TRAF6 mediate NF-κB activation by these receptors (27–36). NF-κB-inducing kinase (NIK) was first identified as a TRAF2-interacting protein and has structural homology to the MAPKKK family (37). Overexpression of NIK-activated NF-κB and a kinase inactive mutant of NIK blocked TNF-, IL-1-, and TRAFs-mediated NF-κB activation, suggesting that NIK is a common downstream mediator of NF-κB activation by TNF, IL-1, and TRAFs (18, 37). TRAF2, TRAF5, and TRAF6 also activate MEKK1, which in turn activates the JNK/SAPK pathway (17, 18). Collectively, these results demonstrate that two responses including NF-κB activation and JNK/SAPK activation diverge downstream of TRAFs.

Recently, three groups (38–42) have independently identified two subunits of the IKK complex, designated IKKα (or IKK-1) and IKKβ (IKK-2) by using a protein purification method or a yeast two-hybrid assay to clone interacting molecules of NIK. Human IKKα, a previously cloned serine-

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Abbreviations: GST, glutathione *S*-transferase; HA, hemagglutinin; IKK, IκB kinase; IL, interleukin; JNK, c-Jun N-terminal kinase; MAPKKK, MAP kinase kinase kinase; MEKK1, mitogen-activated protein kinase/ERK kinase kinase-1; NIK, NF-κB-inducing kinase; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF026524).

‡H.N. and M.S. contributed equally to this work.
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threonine kinase called CHUK (43), and human IKKβ are composed of an N-terminal serine-threonine kinase domain, a central leucine zipper domain, and a C-terminal helix-loop-helix domain. These two kinases show 52% identity at the amino acid level. Biochemical analysis demonstrated that IKKα and IKKβ independently phosphorylate both serine 32 and 36 in IκBα (38–42). Furthermore, overexpression of IKKα or IKKβ activated an NF-κB-dependent reporter and a kinase negative mutant of IKKα- or IKKβ-inhibited NF-κB activation by TNF or IL-1 (38–42). These results clearly demonstrated that the IKKα and IKKβ constitute the functional IKK complex. Although NIK has been shown to activate IKKα (39), the regulation of IKKα and IKKβ kinase activities is still largely unknown.

In the present study, we identified a murine homologue of human IKKβ, which is implicated in NF-κB activation by the TNF-R family members and TRAFs. We demonstrated that overexpression of MEKK1 preferentially stimulated the kinase activity of IKKβ, which resulted in phosphorylation of IκBs. By contrast, overexpression of NIK activated both IKKα and IKKβ comparably, indicating differential regulation of the IKK complex by NIK and MEKK1.

MATERIALS AND METHODS

Reagents and Cell Lines. Anti-Flag mAb and anti-hemagglutinin (HA) mAb (12CA5) were purchased from Kodak International Biotechnology and Boehringer, respectively. The human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum.

cDNA Cloning. To identify an IKKα-related kinase, we searched an expressed sequence tag (EST) database in the National Center for Biotechnology Information (NCBI) DNA database and identified a cDNA clone (AA326115) showing high homology to IKKα. Then, we screened a murine spleen cDNA library (Stratagene) with a PCR fragment corresponding to the EST sequence. Several overlapping clones were obtained and sequenced using series of oligonucleotide primers by standard methods. A full-length IKKα cDNA was obtained by screening the same library by standard methods.

Expression Vectors. Mammalian expression vectors encoding CD27 (C. Morimoto, Institute of Medical Science, University of Tokyo) (44), TRAF2 and CD30 (T. Watanabe, Institute of Medical Science, University of Tokyo) (30), CD40 (H. Kikutani, Research Institute for Microbial Disease, University of Osaka) (45), lymphotoxin-β receptor (LT-βR) (C. F. Ware, La Jolla Institute for Allergy and Immunology) (28), TRAF5 (28), TRAF6, NIK, and NIK-KM(KK429–430AA) (D. Wallach, Weizmann Institute of Science) (37), and MEKK1 and MEKK1-KM(K432M) (S. Ohno, Yokohama City University) (15) have been described previously. Expression vectors for Flag or HA epitope-tagged IKKα and IKKβ were constructed in-frame with DNA encoding an N-terminal Flag or HA epitope in pCR-3 (Stratagene). Expression plasmids encoding IKKα-KM(K44A) and IKKβ-KM(K44A) were generated by using a mutagenesis kit (Stratagene) according to the manufacturer's instruction.

pGEX-IκBα(1–100), pGEX-IκBβ(1–120), and pGEX-IκBe(1–61) were constructed by subcloning the RT-PCR products encoding corresponding amino acids into pGEX-4T vector (Pharmacia). pGEX-IκBα(1–100) (S32A, S36A; designated as 1–100AA), pGEX-IκBβ(1–120) (S19A, S23A; designated as 1–120AA), and pGEX-IκBe(1–61) (S18A, S22A; designated as 1–61AA) were generated by using the mutagenesis kit. Expression and purification of the glutathione *S*-transferase (GST) fusion proteins were performed as described previously (46).

NF-κB-Dependent Reporter Assays. 293 cells (1×10^6) were plated in 35-mm dishes. On the following day, the cells were transfected with the indicated expression vectors using Lipofectamine (Promega). Every transfection included 50 ng of β-actin-β-gal (K. Yokota, NIH, Japan), β-actin promoter-driven β-galactosidase expression plasmid, for the normalization of transfection efficiency, together with 100 ng of the reporter plasmid and various amounts of each expression vector. Total DNA was kept constant by supplementation with pCR-3. The reporter plasmid, 3xκB-L, has three repeats of the NF-κB site upstream of a minimal thymidine kinase promoter and a luciferase gene in pGL-2 vector (Promega) (M. Kashiwada, NIH, Japan). After 24 h, the cells were harvested in PBS and lysed in a luciferase lysis buffer, LC-β (Piccagene, Toyo Ink, Tokyo). The lysates were assayed for luciferase and β-galactosidase activities using a luminometer (Berthold).

***In Vitro* Phosphorylation Assays.** 293 cells (2×10^6) were plated in 60-mm dishes and transfected with various expression vectors using Lipofectamine. After 24–36 h, the cells were washed with ice-cold PBS and lysed for 30 min on ice in 1 ml of a lysis buffer containing 1% Nonidet P-40, 50 mM Hepes (pH 7.3), 150 mM NaCl, 2 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate, and 1 mM NaF. Nuclei were removed by centrifugation, and the supernatant was precleared with protein G-Sepharose (Pharmacia) for 1–2 h. The cleared lysates were incubated with anti-HA or anti-Flag mAb for 1 h at 4°C. After addition of 30 μl of protein G-Sepharose, the lysates were incubated for a further 1 h. The immunoprecipitates were washed three times with the lysis buffer and twice in a kinase buffer containing 20 mM Hepes (pH 7.3), 20 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM EDTA, 1 mM NaF, 0.1 mM sodium orthovanadate, and 1 mM DTT. The immunoprecipitates were then incubated with 1 μg of GST-IκBα(1–100), GST-IκBα(1–100AA), GST-IκBβ(1–120), GST-IκBβ(1–120AA), GST-IκBe(1–61), or GST-IκBe(1–61AA) and [γ-^{32}P]ATP (10 μCi) in the kinase buffer for 20 min at 30°C. The reaction was stopped by addition of the Laemmli's sample buffer. The eluted proteins were subjected to SDS/PAGE, and the autoradiograms were visualized on an image analyzer (Fujix, BAS2000). In all cases, expression of the transfected proteins was verified by immunoblotting of aliquots of the cell lysates as described previously (47). In some experiments, amounts of the GST-IκBs in the reaction mixtures were verified by Coomassic blue staining.

RESULTS AND DISCUSSION

cDNA Cloning and Expression of Murine IKKβ. Recent identification of the first subunit of the IKK complex (IKKα) (38, 39) prompted us to search for IKKα-related kinases. We found a homologous sequence in the EST database and subsequently cloned a full-length cDNA from a murine spleen cDNA library. During preparation of this manuscript, a human kinase highly related to IKKα has been cloned and named IKKβ or IKK-2 (40–42). As our clone has the highest homology to human IKKβ (hIKKβ) (see below), it seems to be the murine IKKβ (mIKKβ).

The mIKKβ cDNA encodes 758 amino acids, which shows 92% and 50% identity to human IKKβ and murine IKKα in amino acid level, respectively (Fig. 1). mIKKβ is composed of an N-terminal serine threonine kinase domain, a leucine zipper domain, and a C-terminal helix-loop-helix domain, as is mIKKα (Fig. 1*B*). Northern blot analysis with the mIKKβ cDNA probe revealed a ubiquitous expression of a 4-kb transcript in various murine tissues (data not shown).

A Kinase Inactive Mutant of IKKβ Blocks NF-κB Activation by Members of the TNF-R Superfamily and TRAFs. A kinase inactive mutant of IKKα or IKKβ blocked TNF- and IL-1-induced NF-κB activation (refs. 39, 41, and 42, and our unpublished results). To test whether IKKβ is also involved in NF-κB activation by other members of the TNF-R superfamily, we constructed a kinase inactive mutant of murine IKKβ

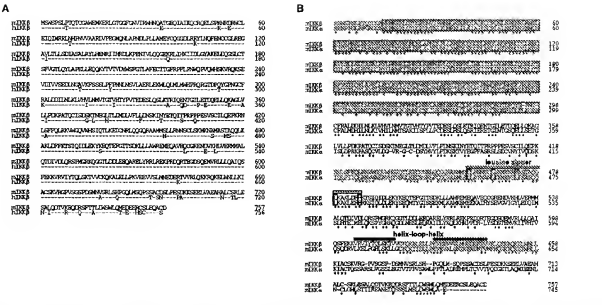

FIG. 1. Amino acid alignment of murine and human IKKα and IKKβ. (*A*) Comparison of murine and human IKKβ. The full-length amino acid sequences are shown and numbered. Dashes in the human sequence indicate residues identical to those in mice. (*B*) Comparison of murine IKKβ and IKKα. Identical residues are indicated by asterisks. The amino-terminal kinase domains are indicated by large boxes, and the conserved amino acids to form a leucine zipper are indicated by small boxes. The leucine zipper and the helix-loop-helix domains are indicated by boldface type.

(IKKβ-KM), in which a lysine at the ATP binding site in the kinase domain was substituted by an alanine. We transiently transfected 293 cells with expression vectors for members of the TNF-R superfamily along with a reporter plasmid, 3xκB-L. As shown in Fig. 2*A*, cotransfection of IKKβ-KM blocked CD27-, CD30-, CD40-, and LT-βR-induced reporter gene activation. NF-κB activation, induced by these receptors, TNF, and IL-1, is mediated by TRAF2, TRAF5, or TRAF6 and their interacting kinase NIK (refs. 18, 25, 28–32, 36, and 37, and our unpublished results). Cotransfection of IKKβ-KM also inhibited NF-κB-dependent reporter gene activity elicited by TRAF2, TRAF5, and TRAF6 (Fig. 2*B*). Furthermore, NF-κB activation by NIK was also inhibited by IKKβ-KM, indicating a critical contribution of IKKβ to the NIK-mediated NF-κB

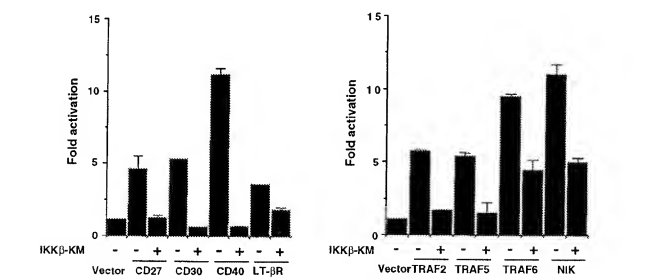

FIG. 2. A kinase inactive mutant of IKKβ (IKKβ-KM) blocks NF-κB activation by members of the TNF-R superfamily or TRAFs. (*A*) Effect of IKKβ-KM on CD27-, CD30-, CD40-, and LT-βR-induced NF-κB-dependent reporter activity. 293 cells were transiently transfected with 100 ng of 3xκB-L and 0.5 μg of expression vectors for CD27, CD30, CD40, or LT-βR along with or without 0.5 μg of IKKβ-KM. Total amount of the DNAs was kept constant by supplementation with pCR-3. The cells were harvested 24 h posttransfection. Luciferase activities were determined and normalized on the basis of β-galactosidase (β-gal) expression from cotransfected β-actin-β-gal (50 ng). The level of induction in luciferase activity was compared as a ration to cells transfected with the control vector. Data are shown as mean ± SEM of triplicated samples and represent one of three experiments with similar results. (*B*) Effect of IKKβ-KM on TRAFs- and NIK-induced NF-κB reporter activity. 293 cells were transfected with 0.5 μg of expression vectors for TRAF2, TRAF5, TRAF6, or NIK with or without 0.5 μg of IKKβ-KM along with 100 ng of 3xκB-L and 50 ng of β-actin-β-gal. Data were obtained and are represented as in *A*.

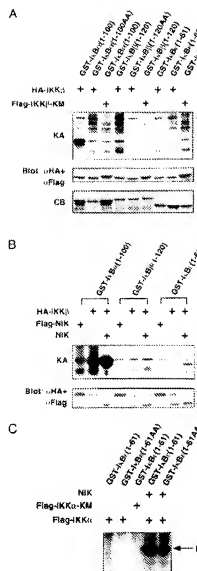

FIG. 3. *In vitro* phosphorylation of IκBα, -β, and -ε by IKKβ and IKKα. (*A*) Specificity of IκBs phosphorylation by IKKβ. 293 cells were transiently transfected with expression vector for HA-IKKβ or Flag-IKKβ-KM. Twenty-four hours after transfection, IKKβ proteins were immunoprecipitated with anti-Flag or anti-HA mAb. The precipitates were incubated with GST-IκBα(1–100), GST-IκBα(1–100) (S32A, S36A; designated as 1–100AA), GST-IκBβ(1–120), GST-IκBβ(1–120) (S29A, S32A; designated as 1–120AA), GST-IκBε(1–61), or GST-IκBε(1–61) (S18A, S22A; designated as 1–61AA) and [γ-^{32}P]ATP, resolved by SDS/PAGE, and analyzed by autoradiography. The kinase activity (KA) is indicated (*Top*). The amounts of IKKβ and IKKβ-KM were determined by immunoblotting with anti-Flag and anti-HA mAbs (*Middle*). The amounts of GST-fusion proteins were assessed by Coomassie Blue (CB) staining (*Bottom*). Right arrows mark the positions of each protein. (*B*) NIK enhances phosphorylation of IκBs by IKKβ. 293 cells were transiently transfected with expression vectors for HA-IKKβ, Flag-NIK, and/or NIK. HA-IKKβ or Flag-NIK was immunoprecipitated and incubated with GST-IκBα(1–100), GST-IκBβ(1–120), or GST-IκBε(1–61) in the presence of [γ-^{32}P]ATP. The kinase activity (KA) is indicated (*Upper*). The amounts of HA-IKKβ and Flag-NIK were determined by immunoblotting with anti-Flag and anti-HA mAbs (*Lower*). The positions of each protein are indicated at the right. (*C*) NIK enhances phosphorylation of IκBε by IKKα. 293 cells were transiently transfected with expression vectors for Flag-IKKα, Flag-IKKα-KM, and/or NIK. Flag-tagged proteins were immunoprecipitated, and *in vitro* phosphorylation of GST-IκBε(1–61) or GST-IκBε(1–61AA) was performed as in *A*. The positions of phosphorylated IKKα and GST-IκBε are indicated (*Right*).

activation, as has been demonstrated for IKKα (39). Collectively, these results indicated that IKKβ is a common downstream kinase for NF-κB activation through members of the TNF-R superfamily and their signal transducers, TRAFs and NIK.

IKKβ Phosphorylates IκBε As Well As IκBα and IκBβ and Is Activated by NIK. To characterize the kinase activity of IKKβ, HA-tagged IKKβ or Flag-tagged IKKβ-KM was transiently expressed in 293 cells. The immunoprecipitates with anti-HA or anti-Flag mAb were subjected to *in vitro* phosphorylation assays using GST fusion proteins of IκBα, β, or ε as substrates. As shown in Fig. 3*A*, IKKβ, but not IKKβ-KM, phosphorylated the wild-type GST-IκBα(1–100) and GST-IκBβ(1–120) but not their mutants, in which the two critical serines for phosphorylation (Ser-32 and Ser-36 for IκBα and Ser-19 and Ser-32 for IκBβ) were both replaced by alanines. The kinase activity of IKKβ to GST-IκBβ was consistently lower than that to GST-IκBα. IκBε is a newly identified member of the IκB family, and phosphorylation of Ser-18 and Ser-22 is required for degradation (7). It has not been determined whether IκBε is also phosphorylated by IKKα or IKKβ. Then, we also examined the phosphorylation of IκBε by IKKα or IKKβ. As shown in Fig. 3 *A–C*, neither IKKβ nor IKKα alone phosphorylated GST-IκBε(1–61).

A previous study demonstrated that NIK stimulates the kinase activity of IKKα to IκBα (39). To test the effect of NIK on the kinase activity of IKKβ, we transfected 293 cells with expression vectors encoding HA-IKKβ along with NIK. The expressed IKKβ was precipitated with anti-HA mAb and subjected to *in vitro* phosphorylation assays. The cotransfected NIK markedly enhanced the phosphorylation of GST-IκBα by IKKβ (Fig. 3*B*). Notably, the coexpression of NIK induced phosphorylation of GST-IκBε(1–61) by IKKβ (Fig. 3*B*). GST-IκBε(1–61) (S18A, S22A; designated as 1–61AA), in which both Ser-18 and Ser-22 were mutated to alanines, was not phosphorylated under the same conditions (data not shown). We next examined whether IKKα could also phosphorylate GST-IκBε(1–61) when coexpressed with NIK. As shown in Fig. 3*C*, NIK stimulated IKKα to phosphorylate GST-IκBε(1–61) but not GST-IκBε(1–61AA). These results indicated that NIK activates IKKβ as well as IKKα and that both IKKα and IKKβ can specifically phosphorylate the critical serine residues of IκBα, IκBβ, and IκBε for their degradation. The kinase activity of IKKs to IκBβ and IκBε seems to be weaker than that to IκBα, which could explain the slower kinetics of degradation of IκBβ and IκBε (7).

NF-κB Activation by MEKK1 Is Mediated by IKKα and IKKβ. NF-κB activation by members of the TNF-R superfamily is mediated by TRAF2, -5, or -6 and their interacting kinase NIK (18, 27–37). On the other hand, these receptors and TRAFs also activate the JNK/SAPK pathway, which is mediated by MEKK1 (17, 18). MEKK1 has been also shown to be involved in TNF-induced NF-κB activation, but the precise mechanism of this pathway remains undefined (14–16). We then examined the contribution of IKKβ and IKKα to the MEKK1-mediated NF-κB activation. We first tested the effect of a catalytically inactive mutant of IKKα or IKKβ on the MEKK1-induced NF-κB activation using reporter assays (Fig. 4). Coexpressed IKKβ-KM or IKKα-KM inhibited the reporter gene activity elicited by MEKK1, indicating that both IKKα and IKKβ are involved in the MEKK1-mediated NF-κB activation. We further examined the contribution of NIK to MEKK1-mediated NF-κB activation. As also shown in Fig. 4, a kinase inactive mutant of NIK (NIK-KM) partially inhibited

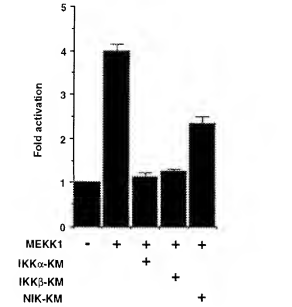

FIG. 4. Effect of IKKβ-KM, IKKα-KM, or NIK-KM on MEKK1-induced NF-κB activation. 293 cells were transiently transfected with 100 ng of 3κB-L and 0.5 μg each of the indicated expression vectors. The NF-κB reporter assays were performed as in Fig. 2. Data represent one of three experiments with similar results.

MEKK1-induced reporter gene activity, suggesting some contribution of NIK to this pathway.

MEKK1 and NIK Differentially Activate IKKα and IKKβ. To further characterize the MEKK1-mediated NF-κB activation pathway, we examined the effect of MEKK1 on the kinase activity of IKKα and IKKβ. As shown in Fig. 5*A*, coexpression of MEKK1, but not MEKK1-KM, markedly enhanced the phosphorylation of GST-IκBα by IKKβ, whereas the IκBα phosphorylation by MEKK1-stimulated IKKα was marginal. In contrast, coexpression of NIK markedly enhanced the kinase activity of both IKKα and IKKβ, and comparable levels of IκBα phosphorylation were observed with NIK-activated IKKα and IKKβ. An apparently greater extent of activation of IKKα than IKKβ by NIK (16.5-fold versus 4.0-fold) is consistent with previous studies (39, 41). These results indicated that MEKK1 preferentially activates IKKβ, whereas NIK efficiently activates both IKKα and IKKβ to phosphorylate IκBα.

Given that overexpressed IKKα or IKKβ most likely forms homodimers, we next examined the effect of NIK or MEKK1 on the kinase activity of IKKαβ heterodimer, which can normally exist in cells (40–42). To form the IKKαβ heterodimer, we transfected Flag-IKKα with or without HA-IKKβ. When IKKα alone was expressed, the immunoprecipitates with anti-Flag mAb phosphorylated GST-IκBα very weakly. In contrast, when Flag-IKKα and HA-IKKβ were coexpressed, phosphorylation of GST-IκBα by the anti-Flag immunoprecipitate was substantially enhanced (Fig. 5*B*), suggesting the coprecipitation of IKKβ with IKKα. In this condition, additional coexpression of either NIK or MEKK1 markedly enhanced the phosphorylation of GST-IκBα, indicating that both NIK and MEKK1 can activate the IKKαβ heterodimer comparably. Taken together, these results suggest that MEKK1 can activate the IKK complex as potently as NIK but in a different manner with a preferential activation of IKKβ. Because NIK has been demonstrated to interact with both IKKα and IKKβ directly (39, 41), the partial inhibition of MEKK1-induced reporter gene activity by NIK-KM (Fig. 4) seems to result from competitive inhibition of MEKK1-mediated activation of IKKβ by overexpressed NIK-KM,

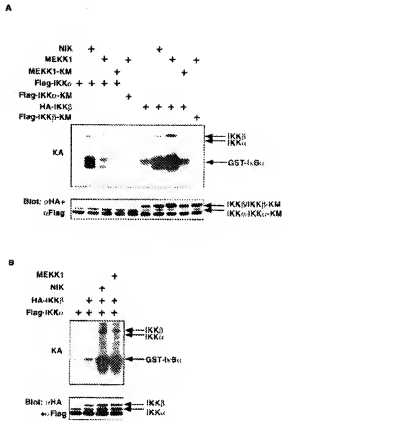

FIG. 5. Regulation of IKKα and IKKβ activities by MEKK1 and NIK. (*A*) Effect of NIK and MEKK1 on IκBα phosphorylation by IKKα or IKKβ. 293 cells were transiently transfected with Flag-IKKα, Flag-IKKα-KM, HA-IKKβ, or Flag-IKKβ-KM along with NIK, MEKK1, or MEKK1-KM. IKKα or IKKβ were immunoprecipitated, and *in vitro* phosphorylation of GST-IκBα(1–100) was performed as in Fig. 3. The kinase activity (KA) is indicated (*Upper*). The amounts of IKKα and IKKβ were determined by immunoblotting with anti-Flag and anti-HA mAbs (*Lower*). The positions of each protein are indicated (*Right*). (*B*) Both NIK and MEKK1 activate IKKαβ heterodimer. 293 cells were transiently transfected with expression vectors for Flag-IKKα and HA-IKKβ along with NIK or MEKK1. IKKα was precipitated with anti-Flag mAb, and *in vitro* phosphorylation of GST-IκBα was performed as in Fig. 3. The kinase activity (KA) is indicated (*Upper*). The amounts of IKKα and IKKβ were determined by immunoblotting with anti-Flag and anti-HA mAbs (*Lower*). The positions of each protein are indicated (*Right*).

rather than a direct contribution of NIK as the downstream kinase of MEKK1. The less efficiency of MEKK1 compared with NIK to activate IKKα also supports this notion and suggests that MEKK1 can activate the IKK complex independently of NIK. In our preliminary experiments, MEKK1 appears not to interact with IKKα or IKKβ directly. However, MEKK1 has been identified to be a component of the large IKK component (42). Therefore, a putative downstream kinase of MEKK1 for IKKβ activation may be involved in the IKK complex, which remains to be identified in the future study.

In the present study, we characterized for the first time the molecular mechanism of the MEKK1-mediated NF-κB activation and found a qualitative difference in the MEKK1- and NIK-mediated NF-κB activation pathways. Our present data are consistent with previous findings that MEKK1 was present in the IKK complex (42) and exogenously added MEKK1 stimulated kinase activity of the IKK complex *in vitro* (14). Various stresses, including UV light, protein synthesis inhib-

ifor, and hyperosmolarity shock, activate both NF-κB and JNK/SAPK pathways. Unlike the TNF case, JNK/SAPK activation by these stresses is not blocked by a dominant negative form of TRAF2, suggesting that this pathway is independent of TRAF and NIK (48). It has been known that these stresses can activate members of the MAPKKK family including MEKK1 (4). Because the activation of MEKK1 by these stresses is independent of TRAF, the presently revealed MEKK1-mediated IKK activation pathway could play a crucial role in NF-κB activation by these stresses. It remains to be determined whether pathways from all stimuli finally converge on IKKα and/or IKKβ, or stimulate other kinases such as $p90^{rsk1}$ (49), for the phosphorylation of IκBs.

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Activation of the IκBα Kinase Complex by MEKK1, a Kinase of the JNK Pathway

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Summary

Both NF-κB and c-Jun are activated by cytokines such as TNF-α and by stresses such as UV irradiation. A key step in the activation of NF-κB is the phosphorylation of its inhibitor, IκBα, by a ubiquitination-inducible multiprotein kinase complex (IκBα kinase). A central kinase in the c-Jun activation pathway is mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1). Here, we show that MEKK1 induces the site-specific phosphorylation of IκBα in vivo and, most strikingly, can directly activate the IκBα kinase complex in vitro. Thus, MEKK1 is a critical component of both the c-Jun and NF-κB stress response pathways. Since the IκBα kinase complex can be independently activated by ubiquitination or MEKK1-dependent phosphorylation, it may be an integrator of multiple signal transduction pathways leading to the activation of NF-κB.

Introduction

Exposure of cells to certain cytokines or environmental stresses leads to the activation of the transcription factors NF-κB and c-Jun. In unstimulated cells, NF-κB is sequestered in the cytoplasm in a complex with IκBα or another member of the IκB family of inhibitor proteins (reviewed by Baldwin, 1996). Following stimulation by a variety of inducers, NF-κB is activated as a consequence of the phosphorylation and subsequent proteolytic degradation of the IκB protein. IκBα is phosphorylated at Serine residues 32 and 36 (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995), an event that targets this protein for degradation by the ubiquitin-proteasome pathway (Chen et al., 1995; reviewed by Baldwin, 1996). Recent studies have identified a large, multisubunit complex in HeLa cell cytoplasmic extracts that can phosphorylate IκBα at Ser-32 and -36 (the IκBα kinase, Chen et al., 1996). A novel property of this kinase, which was isolated from unstimulated cells, is that it can be activated in vitro by ubiquitination.

A second branch of the stress response is the c-Jun N-terminal kinase (JNK) pathway (also known as the stress-activated protein kinase pathway; for recent reviews and references see Karin, 1995, and Kyriakis and Avruch, 1996). The JNK cascade can be activated by small GTP-binding proteins that include Cdc42 and Rac1 (Coso et al., 1995; Minden et al., 1995), and protein kinases that they directly activate, such as PAK (Bagrodia et al., 1995). MEKK1 (Lange-Carter et al., 1993) is then activated by these proteins by a mechanism yet to be determined. MEKK1 then activates MKK4, which in turn activates JNK (Sanchez et al., 1994; Yan et al., 1994; Derijard et al., 1995). Among the substrates of JNK are c-Jun, ATF-2, and Elk-1 (Derijard et al., 1994; Kyriakis et al., 1994; Gupta et al., 1995; Whitmarsh et al., 1995).

While the signal transduction cascade leading to the activation of JNK is relatively well defined, the steps leading to the phosphorylation of IκBα are poorly understood. Many of the stimuli that induce NF-κB, such as TNF-α, UV irradiation, and lipopolysaccharide, also activate the JNK cascade, thereby raising the possibility that the two pathways utilize common signal transduction components. Indeed, transfection of MEKK1 induces the degradation of IκBα and activates an NF-κB reporter gene (Hirano et al., 1996; Meyer et al., 1996), suggesting a link between the NF-κB and JNK pathways. These observations raise two critical questions. First, is MEKK1-induced degradation mediated by the same site-specific phosphorylation of IκBα identified in other contexts? Second, and more importantly, what is the target of MEKK1 in the NF-κB pathway? Here, we show that MEKK1 does in fact induce phosphorylation of IκBα at its sites of regulatory phosphorylation and that this occurs by direct activation of the IκBα kinase. Thus, MEKK1 is a critical coordinate regulator of both the NF-κB and JNK pathways.

Results

IκBα Kinase Activity Is Inducible by TNF-α

Previous studies left open the question of whether the IκBα kinase is regulated by inducers of NF-κB. In those studies, the IκBα kinase was detected as an apparently constitutive activity in S100 cytoplasmic extracts prepared from uninduced HeLa cells using the hypotonic lysis procedure of Dignam et al., 1983 (Chen et al., 1996). Using an alternative method for preparing cytoplasmic extracts (a rapid lysis procedure detailed in Experimental Procedures), we now find that the IκBα kinase activity is inducible by TNF-α. HeLa cells were treated with TNF-α for differing lengths of time, and the rapid lysis extracts assayed for the presence of endogenous IκBα by Western blotting and for IκBα kinase activity by incubation with exogenous ^{35}S-labeled IκBα in the presence of okadaic acid (Figures 1A and 1B). We note that in these and all subsequent experiments okadaic acid is employed strictly as a phosphatase inhibitor (i.e., to preserve the phosphorylated IκBα species) rather than as an inducer of IκBα phosphorylation (Thevenin et al., 1990; Traenckner et al., 1995). Consistent with previous results (Henkel et al., 1993; Mellits et al., 1993), extracts from uninduced HeLa cells contain hypophosphorylated IκBα (Figure 1A, lane 1), but after only 5 min of TNF-α treatment a significant portion of the endogenous IκBα is phosphorylated (as revealed by the slower migrating IκBα species, lane 2). After 30 min of treatment, virtually

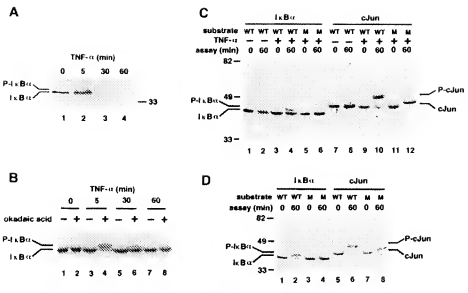

Figure 1. IκBα Kinase Activity Is Inducible and Is Correlated With JNK Activity

(A) HeLa cells were treated with TNF-α for the indicated times, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (14 μg) were then subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-IκBα antibodies. The positions of unphosphorylated (IκBα) and phosphorylated (P-IκBα) IκBα are indicated to the left. Molecular weight marker (in kilodaltons) is indicated to the right.

(B) HeLa cells were treated with TNF-α for the indicated times, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (9 μg) were then incubated with ^{35}S-labeled IκBα in the absence or presence of 6 μM okadaic acid for 1 hr at 30°C. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The positions of unphosphorylated (IκBα) and phosphorylated (P-IκBα) IκBα are indicated to the left.

(C) HeLa cells were either mock or TNF-α (5 min) treated, and cytoplasmic extracts prepared by the rapid lysis procedure. Extracts (8 μg) were then incubated with ^{35}S-labeled wild-type (WT) or mutant (S32A/S36A, M) IκBα, or wild-type (WT) or mutant (S63A/S73A, M) c-Jun for 0 or 60 min at 30°C in the presence of 2.5 μM okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left. The positions of unphosphorylated (cJun) and phosphorylated (P-cJun) c-Jun are indicated to the right; those for IκBα are indicated to the left.

(D) HeLa cell S100 extracts (18 μg) were incubated with ^{35}S-labeled wild-type (WT) or mutant (S32A/S36A, M) IκBα, or wild-type (WT) or mutant (S63A/S73A, M) c-Jun for 0 or 60 min at 30°C in the presence of 2.5 μM okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left.

all of the IκBα is degraded (lane 3). Parallel assays with the same extracts reveal that the IκBα kinase activity is absent in uninduced cells (Figure 1B, lane 2) (activity is weakly detectable when higher concentrations of these extracts are employed). However, after exposure of cells to TNF-α for only 5 min, IκBα kinase activity can be readily detected, as evidenced by the slower-migrating IκBα species (lane 4). Fractionation of these extracts by gel filtration reveals that the TNF-α–inducible IκBα kinase activity resides in a large (approximately 700 kDa) complex (data not shown). Interestingly, this activity persists and is present after 30 min of TNF-α induction (lane 6), a time at which the endogenous IκBα has been degraded (Figure 1A, lane 3). IκBα kinase activity is essentially absent at 60 min (Figure 1B, lane 8). It is formally possible that the IκBα kinase activity is constitutive and that TNF-α treatment simply results in the inactivation of a phosphatase in the extract that dephosphorylates IκBα. To address this possibility, the same extracts were incubated with ^{35}S-labeled IκBα in the absence of okadaic acid (Figure 1B, lanes 1, 3, 5, and 7). Under these conditions, the IκBα mobility shift is completely abolished (for example, compare lanes 3 and 4). Thus, the effects of TNF-α treatment cannot be accounted for solely by inactivation of an okadaic acid–sensitive IκBα phosphatase, implying that TNF-α treatment induces IκBα kinase activity. Furthermore, the rapid induction of IκBα kinase activity correlates with the rapid appearance of the phosphorylated form of IκBα.

Coordinate Activation of IκBα Kinase and JNK Activities In Vitro

TNF-α treatment also leads to the activation of c-Jun by JNK (Hibi et al., 1993). We therefore carried out experiments to determine whether the IκBα kinase and JNK are coactivated in extracts from TNF-α–treated cells. Cytoplasmic extracts from uninduced and TNF-α–induced HeLa cells were incubated with in vitro translated, ^{35}S-labeled IκBα or c-Jun, and the proteins fractionated by SDS-PAGE (Figure 1C). As before, IκBα kinase activity is detected in extracts from TNF-α–induced cells but not in those from uninduced cells (compare lanes 2 and 4). The specificity of phosphorylation is indicated by the fact that the S32A/S36A mutation in IκBα completely abolishes the IκBα shift (lane 6). Similarly, extracts from TNF-α–induced cells show JNK activity, as evidenced by the appearance of a c-Jun species with markedly reduced mobility (compare lanes

9 and 10). The observed shift is a result of JNK activity, since amino acid substitutions at the sites of JNK phosphorylation (S63A/S73A) in c-Jun abolish this shift (lane 12). We note that a distinct shift is observed with the c-Jun mutant, suggesting that JNK may phosphorylate c-Jun at residues other than Ser-63 and -73. Importantly, extracts from uninduced cells show no significant JNK activity (lane 8). Thus, both the IκBα kinase and JNK activities are activated in the rapid lysis extracts prepared from TNF-α-treated, but not untreated, cells.

By contrast, the IκBα kinase activity is readily detected in S100 cytoplasmic extracts prepared from unstimulated HeLa cells using the hypotonic lysis procedure (Chen et al., 1995). It is possible that stress pathways are activated by this procedure, since another form of osmotic stress, hyperosmolar shock, has been shown to be an efficient activator of the JNK pathway (Galcheva-Gargova et al., 1994). Indeed, both the IκBα kinase and the JNK activities were detected when the S100 extracts were incubated for 60 min (Figure 1D). A time-dependent activation of JNK was detected when the S100 extracts were incubated and then examined by an in-gel kinase assay employing the JNK substrate ATF-2 (data not shown). Thus, both the JNK and IκBα kinase may be activated during incubation of the S100 extracts, possibly owing to the hypotonic lysis conditions.

MEKK1 Activates NF-κB In Vivo

Transient transfection studies were conducted to examine the relationship between the activation of the IκBα kinase and JNK in vivo. The IFN-β enhancer contains multiple positive regulatory domains (PRDs) that bind distinct transcription factors, including NF-κB (PRDII) and ATF-2/c-Jun (PRDIV) (reviewed in Thanos et al., 1993). HeLa cells were transfected with reporters linked to either two copies of PRDII (PII), six copies of PRDIV (PIV), or the intact IFN-β enhancer (IFN), which includes these as well as other PRDs, and either an expression vector for MEKK1 or an expression vector alone. Note that in these and all subsequent experiments, MEKK1 and MEKK1Δ refer to the 672 and 321 residue C-terminal fragments, respectively, of the full-length molecule (for discussion, see Xu et al., 1996). Both kinases are constitutively active and indistinguishable in transfection studies. As expected, MEKK1 activates the reporter linked to a multimer of PRDIV (Figure 2A), which binds to either an ATF-2 homodimer or an ATF-2/c-Jun heterodimer (Du et al., 1993). Both ATF-2 and c-Jun contain transcriptional activation domains that are phosphorylated by the JNK pathway (Gupta et al., 1995). Importantly, MEKK1 also activates the PRDII reporter. MEKK1 does not activate all promoters, since its effect on a reporter gene containing the intact IFN-β enhancer is only marginal. This enhancer contains additional PRDs that bind factors other than NF-κB or ATF-2/c-Jun (see Thanos et al., 1993). As expected, the IFN-β enhancer is effectively activated by virus infection, which activates all of the PRDs. We conclude that MEKK1 can activate both ATF-2/c-Jun and NF-κB in vivo.

To examine whether MEKK1 plays a role in the activation of NF-κB in response to TNF-α, HeLa cells were transfected with a PRDII reporter and expression vector for catalytically inactive (K432M) MEKK1Δ, or empty expression vector. Some cells were then stimulated with TNF-α, and subsequently all cells were harvested and examined for reporter gene activity. As expected, TNF-α activates the PRDII reporter efficiently (Figure 2B). By contrast, the mutant MEKK1Δ (K432M) inhibits both the basal and TNF-α-induced activity of this reporter, thus behaving as a dominant negative inhibitor, as has also been shown by Hirano et al. (1996). Similar results are observed in L929 cells (Figure 2C). As a negative control, cAMP activation of a cAMP response element reporter is not significantly affected by dominant negative MEKK1Δ (Figure 2D). These results suggest that MEKK1 plays a role in TNF-α activation of NF-κB.

MEKK1 Activation of NF-κB Occurs through Site-Specific Phosphorylation of IκBα

Numerous stimuli that activate NF-κB have been shown to induce site-specific phosphorylation of IκBα at Ser-32 and -36 (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995). Experiments were therefore conducted to examine whether MEKK1 induces this same phosphorylation. HeLa cells were transfected with

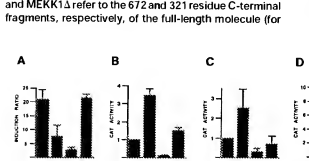

Figure 2. MEKK1 Activates NF-κB In Vivo

(A) HeLa cells were transfected with 3 μg of $(PRDII)_2$CAT, $(PRDIV)_4$CAT or -110IFN-βCAT, 2 μg pCMV-lacZ, and 4 μg of pCMV5-MEKK1 or pcDNA3. Twenty-six to 28 hr posttransfection, cells in one well were infected with Sendai virus for 15 hr. All cells were harvested 41 to 43 hr posttransfection. CAT activities were normalized to protein concentrations of cell extracts. Shown are the averages and standard deviations from three independent experiments.

(B and C) HeLa (B) or L929 (C) cells were transfected with 3 μg of $(PRDII)_2$CAT, 2 μg pCMV-lacZ, and 4 μg of pcDNA3-FlagMEKK1Δ (K432M) or pcDNA3. Forty to 41 hr posttransfection, some cells were treated with 20 ng/ml mouse TNF-α (Boehringer) for 8 hr. All cells were harvested 48 to 49 hr posttransfection. CAT activities were normalized to those for β-galactosidase. Shown are the averages and standard deviations from (B) one experiment performed in triplicate or (C) three independent experiments.

(D) L929 cells were transfected with 3 μg of $(CRE)_4$CAT, 2 μg pCMV-lacZ, and 4 μg of pcDNA3-FlagMEKK1Δ (K432M) or pcDNA3. Forty to 41 hr posttransfection, some cells were treated with 1 mM 8-Br-cAMP for 8 hr. All cells were harvested 48 to 49 hr posttransfection. CAT activities were normalized to those for β-galactosidase. Shown are the averages and standard deviations from three independent experiments.

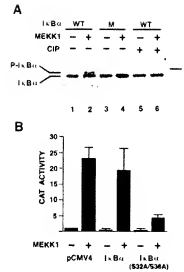

Figure 3. MEKK1 Activation of NF-κB Isthrough Site-Specific Phosphorylation of IκBα

(A) HeLa cells were transfected with 0.3 μg of expression vectors for wild-type (WT) (pCMV4-FlagIκBα) or mutant (M) (pCMV4-FlagIκBα [S32A/S36A]) IκBα, 3 μg of pCMV5-MEKK1 or pCMV5, and 3 μg of SP72. Forty-one hr posttransfection, epitope-tagged IκBα was immunoprecipitated, and some samples were treated with calf intestinal alkaline phosphatase (CIP). All samples were then subjected to 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-IκBα antibodies.

(B) HeLa cells were transfected with 3 μg of (PRDII)$_2$CAT, 10 ng of pCMV4-FlagIκBα, pCMV4-FlagIκBα (S32A/S36A) or pCMV4, and 4 μg of pCMV5-MEKK1 or pCMV5. Cells were harvested 38 to 43 hr posttransfection. CAT activities were normalized to protein concentrations of cell extracts. Shown are the averages and standard deviations from three independent experiments.

expression vectors for Flag-tagged wild-type or mutant (S32A/S36A) IκBα, and an expression vector for MEKK1 or the expression vector alone. IκBα was then immunoprecipitated with anti-Flag antibodies, and then visualized by Western blotting using anti-IκBα antibodies. MEKK1 induces the appearance of an IκBα species with reduced mobility compared to that isolated from uninduced cells (Figure 3A, lane 2). This species is sensitive to treatment with calf intestinal alkaline phosphatase (compare lanes 2 and 6), consistent with its being a phosphorylated form of IκBα. Most importantly, Ser-to-Ala mutations at residues 32 and 36 of IκBα abolish this species (lane 4). Thus, MEKK1 induces site-specific phosphorylation of IκBα at Ser-32 and -36.

If the MEKK1-dependent phosphorylation of these serine residues is functionally important, their substitution by alanines should make IκBα degradation and hence NF-κB activation refractory to MEKK1 stimulation. To test this possibility, HeLa cells were transfected with the PRDII reporter gene and expression vectors for wild-type or mutant (S32A/S36A) IκBα or expression vector alone and either expression vector for MEKK1 or expression vector alone. HeLa cells transfected with wild-type IκBα display MEKK1-inducible reporter gene activity that is virtually the same as that of control (Figure 3B). In contrast, HeLa cells transfected with mutant IκBα display MEKK1-inducible activity that is significantly diminished. Other experiments indicate that it is necessary to transfect approximately 10-fold higher amounts of wild-type IκBα plasmid in order to observe a comparably reduced MEKK1-inducible reporter gene activity (data not shown). These experiments therefore indicate that site-specific phosphorylation of IκBα at Ser-32 and -36 plays a critical role in the MEKK1-dependent activation of NF-κB.

MEKK1 Coordinately Activates the IκBα Kinase and JNK In Vitro

The transfection data show that MEKK1 expression leads to the site-specific phosphorylation of IκBα. To investigate the possibility that MEKK1 activates the IκBα kinase, cytoplasmic extracts were prepared from uninduced HeLa cells by the rapid lysis procedure and then treated with recombinant MEKK1Δ. In the absence of MEKK1Δ, these extracts show no significant site-specific IκBα kinase or JNK activity when incubated with in vitro-translated, ^{35}S-labeled IκBα or c-Jun, respectively (Figure 4A, lanes 2 and 6). By contrast, when recombinant MEKK1Δ was added to the extract, site-specific phosphorylation of c-Jun was observed (compare lanes 7 and 9). Importantly, site-specific IκBα kinase activity was also observed (compare lanes 3 and 5), but MEKK1Δ alone fails to induce this site-specific phosphorylation (lane 1). To rule out the possibility that MEKK1Δ inactivates an IκBα phosphatase, extracts were incubated with MEKK1Δ in the absence or presence of the phosphatase inhibitor okadaic acid (Figure 4B). In the absence of okadaic acid, the MEKK1Δ-induced IκBα shift is largely abolished (compare lanes 2 and 3). Thus, the effects of MEKK1Δ cannot be accounted for solely by the inactivation of an okadaic acid–sensitive IκBα phosphatase, implying that MEKK1Δ activates the IκBα kinase. We conclude that MEKK1Δ coordinately activates the IκBα kinase and JNK pathways in cytoplasmic extracts.

MEKK1 Directly Activates the IκBα Kinase

In the JNK pathway, MEKK1 phosphorylates and activates MKK4, which, in turn, activates JNK. It is therefore possible that IκBα could be a substrate for MEKK1, MKK4, or JNK. When expressed as recombinant proteins, however, neither MKK4 nor JNK1 phosphorylated IκBα, with appropriate control experiments demonstrating that these proteins were enzymatically active (data not shown). MEKK1Δ did phosphorylate IκBα directly; however, the degree of phosphorylation was over 10-fold less than that seen with MKK4 as a substrate, and, as shown below, MEKK1Δ does not phosphorylate IκBα at Ser-32 or -36. In addition, recent experiments indicate the IκBα kinase activity resides in a large, approximately 700 kDa, multiprotein complex (Chen et al., 1996), and Western blotting of this complex fails to reveal the presence of MEKK1, MKK4, JNK1, or JNK2 (data not shown). A reasonable hypothesis, therefore, is that MEKK1 or one of the downstream kinases phosphorylates IκBα indirectly by stimulating the IκBα kinase.

To distinguish between these possibilities, MEKK1Δ was incubated with purified, ubiquitination-inducible

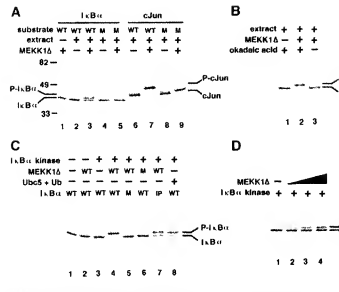

Figure 4. MEKK1 Directly Activates the IκBα Kinase

(A) Uninduced HeLa cell cytoplasmic extracts (2 μg) prepared by the rapid lysis procedure were incubated with ^{35}S-labeled wild-type (WT) or mutant (S32A/S36A, M) IκBα, or wild-type (WT) or mutant (S63A/S73A, M) c-Jun in the absence or presence of 20 ng MEKK1Δ for 1 hr at 30°C in the presence of 2.5 μM okadaic acid. An additional incubation (lane 1) contained 20 ng MEKK1Δ and ^{35}S-labeled IκBα in the absence of extract. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kilodaltons) are indicated to the left.

(B) Uninduced HeLa cell cytoplasmic extracts (2 μg) prepared by the rapid lysis procedure were incubated with ^{35}S-labeled IκBα in the absence or presence of 20 ng MEKK1Δ and/or 2.5 μM okadaic acid for 1 hr at 30°C. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

(C) Purified IκBα kinase was incubated with wild-type (WT), mutant (S32A/S36A) (M), or immunoprecipitated wild-type (IP) ^{35}S-labeled FlagIκBα in the absence or presence of 20 ng wild-type (WT) or mutant (K432M) (M) MEKK1Δ, or 0.9 μg GST-Ubc5 + 0.5 mg/ml ubiquitin for 1 hr at 30°C in the presence of 2.5 μM okadaic acid. An additional incubation (lane 1) contained 20 ng MEKK1Δ and ^{35}S-labeled FlagIκBα in the absence of IκBα kinase. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. In lane 8, additional bands at higher molecular weights than phosphorylated IκBα represent ubiquitinated IκBα species, owing to the presence of ubiquitination components (Chen et al., 1995, 1996).

(D) Purified IκBα kinase in the absence or presence of 5, 10, or 20 ng MEKK1Δ was incubated with ^{35}S-labeled FlagIκBα for 1 hr at 30°C in the presence of 2.5 μM okadaic acid. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

IκBα kinase (Chen et al., 1996) and in vitro–translated, ^{35}S-labeled IκBα (Figure 4C). In the absence of the ubiquitin-conjugating enzyme Ubc5 and ubiquitin, the IκBα kinase is inactive (lane 3), while in their presence the kinase is active, as evidenced by the shift in mobility of the ^{35}S-labeled IκBα (lane 8), as shown previously (Chen et al., 1996). Strikingly, addition of MEKK1Δ independently activates the IκBα kinase (lane 4), while MEKK1Δ alone does not site-specifically phosphorylate IκBα (lane 2). That this shift reflects phosphorylation of IκBα at Ser-32 and -36 is indicated by the fact that the S32A/S36A mutant fails to display this shift (lane 5). The activation of the IκBα kinase depends on the catalytic activity of MEKK1Δ, since mutant MEKK1Δ(K432M) fails to activate (lane 6). Neither recombinant MKK4 nor JNK1 augments MEKK1Δ stimulation of the IκBα kinase (data not shown), ruling out the possibility that MEKK1Δ activation of the IκBα kinase is mediated by an insect (Sf9) cell MKK4- or JNK-like activity copurifying in trace amounts with the MEKK1Δ protein. To eliminate the possibility that MEKK1Δ activation of the IκBα kinase is mediated through a factor present in the wheat germ extract employed for in vitro translation of IκBα, immunoprecipitated IκBα was employed as a substrate. As shown in lane 7, this IκBα is also a substrate for MEKK1Δ-activated IκBα kinase. We conclude that MEKK1Δ activation of the IκBα kinase is direct. Additional experiments indicate that MEKK1Δ is a potent activator of the IκBα kinase (Figure 4D), with activation demonstrable with MEKK1Δ doses as low as 5 ng (lane 2). Finally, IκBα complexed with RelA (p65) is a substrate for MEKK1Δ-activated IκBα kinase, just as it is for the ubiquitination-activated kinase (data not shown).

The MEKK1-Inducible IκBα Kinase Is a High Molecular Weight Complex

To further examine the relationship between the MEKK1Δ- and the ubiquitination-inducible IκBα kinase previously reported (Chen et al., 1996), we fractionated HeLa cell cytoplasmic extracts and assayed for both activities (Figure 5). Notably, both MEKK1Δ- and ubiquitination-inducible IκBα kinase activities copurify during the first four steps of fractionation, which include ion exchange chromatography, ammonium sulfate fractionation, hydroxylapatite chromatography (data not shown), and gel filtration (Figures 5A and 5B). With regard to the gel filtration step, the peak of MEKK1Δ-inducible IκBα kinase activity elutes at a position (fractions 19 to 20) corresponding to a native molecular weight of approximately 700 kDa, indistinguishable from that of the ubiquitination-inducible IκBα kinase (Chen et al., 1996). Further fractionation by anion exchange chromatography reveals that the MEKK1Δ-inducible IκBα kinase activity elutes in a broader peak than the ubiquitination-inducible activity (Figures 5C and 5D). Thus, some fractions (e.g., 32 and 33) are inducible by both MEKK1Δ and ubiquitination, while others (e.g., 29 and 30) are inducible only by MEKK1Δ. We conclude that the two kinase complexes are largely similar but may have subtle differences in structure or subunit composition.

MEKK1 Is a Selective Activator of the IκBα Kinase

To examine the specificity of MEKK1Δ activation of the IκBα kinase, three additional kinases, casein kinase II (CKII), protein kinase A (PKA), and protein kinase Cζ (PKCζ), were assayed for their capacity to activate the

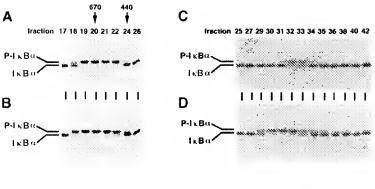

Figure 5. The MEKK1-Inducible IκBα Kinase Is a High Molecular Weight Complex

HeLa cell cytoplasmic extracts were fractionated as described in Experimental Procedures and then chromatographed on (A and B) a Superdex-200 gel filtration column followed by a (C and D) Mono-Q ion exchange column. Fractions were assayed for IκBα kinase activity with ^{35}S-labeled FlagIκBα in the presence of either (A and C) ubiquitination components or (B and D) 10 ng MEKK1Δ for 1 hr at 37°C in the presence of 3 μM okadaic acid. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The numbers 670 and 440 in (A) and (B) indicate elution positions of molecular weight standards (in kilodaltons).

IκBα kinase (Figure 6). In marked contrast to MEKK1Δ, none of these enzymes activates the IκBα kinase (Figure 6A). The enzymatic activity of the kinases is demonstrated by their roughly comparable degree of phosphorylation of recombinant IκBα with [γ-^{32}P]ATP (Figure 6B). The experiment shown in Figure 6A also demonstrates that none of the enzymes, aside from the IκBα kinase, phosphorylates IκBα at Ser-32 or -36 under the conditions employed. Phosphorylation by these other enzymes presumably occurs at residues other than Ser-32 or -36.

MEKK1 Activates the IκBα Kinase Complex by Phosphorylation

The fact that the catalytically inactive MEKK1Δ does not activate the IκBα kinase (Figure 4C, lane 6) strongly suggests that MEKK1Δ phosphorylates the IκBα kinase complex. To further examine this possibility, MEKK1Δ-activated IκBα kinase was incubated with or without calf intestinal alkaline phosphatase, and the IκBα kinase was then assayed for activity against ^{35}S-labeled IκBα in the absence or presence of MEKK1Δ. As shown in Figure 7A, treatment of the MEKK1Δ-activated IκBα kinase with phosphatase results in inactivation of IκBα kinase activity (compare lanes 2 and 4). Subsequent addition of MEKK1Δ results in substantial, though incomplete, restoration of IκBα kinase activity (compare lanes 2, 3, and 4). To extend these observations, the purified IκBα kinase was incubated with or without MEKK1Δ in the presence of [γ-^{32}P]ATP (Figure 7B). In the absence of MEKK1Δ, ^{32}P label was incorporated into three subunits (approximately 200, 180, and 120 kDa) of the IκBα kinase complex (lane 2). In the presence of MEKK1Δ, ^{32}P label was incorporated into three additional subunits of molecular weights of approximately 105, 64, and 54 kDa (lane 3). In conjunction with the experiment employing the catalytically inactive MEKK1Δ (Figure 4C), these experiments show that MEKK1Δ activates the IκBα kinase complex by phosphorylation. We are currently investigating which subunit(s) is the substrate(s) for MEKK1.

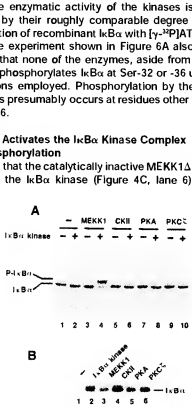

Figure 6. MEKK1 Is a Selective Activator of the IκBα Kinase

(A) MEKK1Δ (10 ng), CKII (0.35 ng, 250 mU, New England Biolabs), PKA (0.8 ng, 1 mU, New England Biolabs), and PKCζ (15 ng, PanVera), either alone or in combination with purified IκBα kinase, were incubated with ^{35}S-labeled FlagIκBα for 30 min at 30°C in the presence of 2.5 μM okadaic acid. An additional incubation (lane 2) contained purified IκBα kinase and ^{35}S-labeled FlagIκBα. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography.

(B) Purified IκBα kinase, MEKK1Δ, CKII, PKA, and PKCζ in the amounts employed in (A) were incubated with 0.5 μg (His)$_6$IκBα in the presence of [γ-^{32}P]ATP. Reaction products were subjected to 10% SDS-PAGE and analyzed by autoradiography. Relative kinase activities determined by phosphonmager analysis for the IκBα kinase, MEKK1Δ, CKII, PKA, and PKCζ are 1, 0.6, 2.2, 1.0, and 1.3, respectively.

Discussion

The activation of the IκBα kinase and JNK pathway by a single protein, MEKK1, provides a compelling explanation for how multiple stimuli can simultaneously activate these two distinct kinases (Figure 8). Thus, TNF-α, UV irradiation, and lipopolysaccharide have all been shown to activate the JNK pathway. Their implied activation of MEKK1 now provides a mechanism for the activation of the IκBα kinase. Stimuli such as phorbol myristate acetate/ionomycin could also potentially act through this pathway; in T cells, for example, phorbol myristate acetate and ionomycin synergistically activate the JNK pathway (Su et al., 1994) and thus may activate the IκBα kinase through MEKK1. The coordinate activation of the IκBα kinase and JNK raises the possibility that potential upstream activators of MEKK1, such as the small GTP-binding proteins Rac1, Cdc42, and Ras, as well as protein kinases that they activate, such as PAK, may also be common elements of a single upstream signal transduction mechanism.

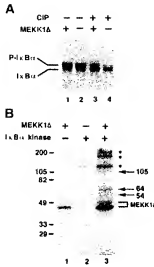

Figure 7. MEKK1 Activates the IκBα Kinase Complex by Phosphorylation

(A) MEKK1Δ-activated IκBα kinase was incubated with or without calf intestinal alkaline phosphatase (CIP, as indicated), and subsequently incubated with or without 12 ng MEKK1Δ (as indicated) and with ^{35}S-labeled FlagIκBα for 60 min at 37°C in the presence of 3 μM okadaic acid. Reaction products were subjected to 9% SDS-PAGE and analyzed by autoradiography. The doublet above the IκBα probably represents phosphorylation at one or both serines at positions 32 and 36.

(B) MEKK1Δ and purified IκBα kinase, either alone or in combination, were incubated in the presence of [γ-^{32}P]ATP. Reaction products were subjected to 8% SDS-PAGE and analyzed by autoradiography. Molecular weight markers (in kDa) are shown to the left. Dots indicate bands (approximately 200, 180, and 120 kDa) present when the IκBα kinase is incubated with [γ-^{32}P]ATP in the absence of MEKK1Δ. Bracket indicates bands present when MEKK1Δ is incubated with [γ-^{32}P]ATP in the absence of the IκBα kinase, showing MEKK1Δ autophosphorylation.

Previous studies have implicated kinases other than MEKK1 in the activation of NF-κB. For example, PKA has been shown to dissociate the NF-κB–IκB complex (Ghosh and Baltimore, 1990), while PKCζ coimmunoprecipitates with a factor that can phosphorylate IκBα (Diaz-Meco et al., 1994); with regard to the latter, it has been suggested that PKCζ activates a kinase that phosphorylates IκBα. Neither PKA nor PKCζ, however, phosphorylates IκBα at Ser-32 and-36, nor does either activate the IκBα kinase (Figure 6A). Additional kinases that have been implicated in NF-κB activation are raf-1 and the double-stranded RNA-activated protein kinase (PKR) (Finco and Baldwin, 1993; Yang et al., 1995). In preliminary experiments, we have been unable to observe activation of the IκBα kinase by enzymatically active c-raf (UBI) (data not shown). Additional studies will be required to determine the roles of raf and PKR in NF-κB regulation.

MEKK1 is a member of a family of enzymes that share a conserved C-terminal catalytic domain and may thus share overlap in substrates (Lange-Carter et al., 1993; Blank et al., 1996; Xu et al., 1996). Hence, it is conceivable that MEKK isoforms other than MEKK1 can activate the IκBα kinase. Different MEKK isoforms could potentially be involved in signaling responses to different stimuli. For example, MEKK1 has been shown to bind Ras

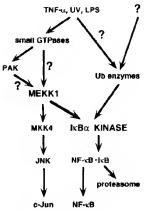

Figure 8. Model Showing Coordinate Activation of the IκBα Kinase and the JNK Pathway by MEKK1

Stressful stimuli such as TNF-α, UV irradiation, and lipopolysaccharide (LPS) activate MEKK1 by as-yet-to-be-identified mechanisms that involve small GTPases such as Rac1, Cdc42, and Ras and possibly the protein kinase PAK. MEKK1, in turn, coordinately activates the JNK pathway—which ultimately phosphorylates c-Jun—and the IκBα kinase. The IκBα kinase phosphorylates IκBα while still bound to NF-κB, targeting IκBα for degradation by the ubiquitin-proteasome pathway and thereby liberating NF-κB. The IκBα kinase can independently be activated by ubiquitination (Ub) enzymes (see Chen et al., 1996).

in a GTP-dependent manner and thus its activity may be regulated in a similar fashion (Russell et al., 1995). Indeed, Ha-Ras activation of NF-κB (Devary et al., 1993) could be mediated, in part, by activation of MEKK1. Aside from its interaction with Ras, little is known about the regulation and activation of MEKK1. The recent identification of MEKK1 as a large membrane-associated protein, with its C-terminal catalytic domain constituting less than 20% of the molecule, raises the possibility of complex modes of regulation (Xu et al., 1996). While yet to be demonstrated, other MEKKs could conceivably be regulated by other upstream regulators such as Rac1, Cdc42, and PAK. These data also leave open the possibility that there may be other IκBα kinases that respond to stimuli distinct from those that signal through MEKK1.

We as well as others (Hirano et al., 1996) have shown that dominant negative MEKK1 inhibits TNF-α activation of an NF-κB reporter gene in vivo. This is in contrast to a recent report that reveals no effect of dominant negative MEKK1 in similar experiments, which conclude that MEKK1 lies on a pathway distinct from that of the IκBα kinase (Liu et al., 1996). At present, we have no explanation for this discrepancy. However, our transfection results are strongly supported by the observation that the IκBα kinase is phosphorylated and activated by MEKK1Δ in vitro. We thus conclude that the IκBα kinase and MEKK1, or minimally an MEKK isoform, are indeed part of the same pathway.

The IκBα kinase can be activated by ubiquitination independently of phosphorylation (Figure 8). To our knowledge, this dual regulation by phosphorylation or ubiquitination is unprecedented. Thus, the IκBα kinase itself can be considered a signal integrator, responding to both phosphorylation and ubiquitination. Different stimuli may therefore activate one, the other, or both

pathways. In principle, then, it may be possible to isolate an induced IκBα kinase species that is not ubiquitination-dependent, or one that is not phosphorylation-dependent. Indeed, the fractionation of highly purified IκBα kinase by ion exchange chromatography reveals kinase species that are phosphorylation but not ubiquitination inducible (Figures 5C and 5D).

A puzzling result from previous studies is that while IκBα kinase is easily assayed when present in HeLa cell S100 cytoplasmic extracts, the purified kinase is inactive, requiring ubiquitination components for activity (Chen et al., 1996). One possibility is that purification of the kinase separates the ubiquitination components from the kinase; hence, the purified kinase is inactive. The results described here raise a second and distinct possibility, namely that the IκBα kinase is activated by MEKK1 in the extract during the course of assay for IκBα kinase activity. Thus, purification of the IκBα kinase from S100 extracts removes it from both the ubiquitination components and MEKK1 present in the extract; in fact, Western blotting indicates that MEKK1 is not present in the IκBα kinase complex (data not shown). Either ubiquitination or MEKK1-dependent phosphorylation can activate the purified IκBα kinase (Figure 4C).

The detailed molecular mechanism by which MEKK1 activates the IκBα kinase remains to be determined. One possibility is that MEKK1 inactivates a negative regulatory subunit of the IκBα kinase, just as cAMP binds to and induces the dissociation of the regulatory subunit of PKA (Francis and Corbin, 1994). Alternatively, MEKK1 may activate the catalytic subunit of the IκBα kinase that subsequently phosphorylates Ser-32 and -36 of IκBα. Yet another possibility is that MEKK1 initiates a MAPK-like cascade within the IκBα kinase complex, with the terminal kinase the subunit that phosphorylates Ser-32 and -36; this would be somewhat analogous to the organization of MAPK modules as high molecular weight complexes in yeast (Choi et al., 1994). The incorporation of ^{32}P into multiple subunits of the IκBα kinase complex in the presence of MEKK1Δ (Figure 7) could be consistent with any of these possibilities. It will be of interest to determine if the IκBα kinase subunit(s) targeted by MEKK1 is the same as those targeted by the ubiquitination system. Further conclusions must await the identification and characterization of subunits of the IκBα kinase.

Experimental Procedures

Plasmids

pCMV5-MEKK1 (which encodes the C-terminal 672 residues of MEKK1), pcDNA3-FlagMKK4, and pSRαHA-JNK1 were gifts of Dr. Roger Davis (University of Massachusetts, Worcester) and have been described (Derijard et al., 1994, 1995; Whitmarsh et al., 1995). pcDNA3-MEKK1 was constructed by subcloning the 2.4 kb EcoRI/EcoNI (blunt) fragment of pCMV5-MEKK1 encoding MEKK1 into the EcoRI/EcoRV site of pcDNA3. pcDNA3-FlagMEKK1Δ (K432M) consists of an N-terminal Flag epitope fused to the C-terminal 321 amino acid fragment of MEKK1 with the indicated mutation (amino acid numbering according to Lange-Carter et al., 1993) and was constructed by polymerase chain reaction (Ausubel et al., 1989). pcDNA3-FlagMEKK1Δ was constructed by replacing the 2.1 kb StuI fragment of pcDNA3-FlagMEKK1Δ (K432M), which encodes the C-terminal 262 amino acids with the corresponding fragment of pcDNA3-MEKK1. pCMV4-FlagIκBα and pCMV4-FlagIκBα(S32A/S36A) were gifts of Dr. Dean Ballard (Vanderbilt University) and have been described (Brockman et al., 1995). pcDNA1-c-Jun has been described (Du et al., 1993). pcDNA1-c-Jun(S63A/S73A) was constructed using overlapping polymerase chain reaction (Ausubel et al., 1989). pBS-IκBα, pBS-IκBα(S32A/S36A), pBS-FlagIκBα, pBS-FlagIκBα(S32A/S36A), $(PRDII)_4$CAT, $(PRDIV)_4$CAT, $(CRE)_4$CAT, −110IFN-βCAT, and pCMV-lacZ have been described (MacGregor and Caskey, 1989; Du and Maniatis, 1992; Thanos and Maniatis, 1992; Chen et al., 1995).

Tissue Culture and Transfection

HeLa and L929 cells were maintained in DME media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Transfections and virus infections, performed in 3.5 cm diameter wells, were conducted as described (Thanos and Maniatis, 1992). Cells were typically harvested at 41 to 49 hr posttransfection. CAT and β-galactosidase assays were performed as described (Sambrook et al., 1989). Protein concentrations were measured by the Bradford method.

Immunoprecipitations

Transfected IκBα

Cell pellets obtained by harvesting 3.5 cm diameter wells were lysed by the addition of 200 μl of Buffer A (20 mM Tris, pH 7.5, 0.4 M KCl, 4 mM β-glycerolphosphate, 0.1 mM sodium orthovanadate, 0.1% NP-40, 10% glycerol, 10 μg/ml leupeptin, 1 mM PMSF, and 1 mM DTT), followed by three freeze/thaw cycles. After centrifugation at 14,000 × g for 5 min at 4°C, the supernatant (320 μg protein) was incubated with 20 μl of M2-agarose (IBI-Kodak) in 1 ml of Buffer A with end-over-end rotation for 1 hr at 4°C. Resins were then washed three times with Buffer A and once with 0.1X Buffer A.

^{35}S-Labeled FlagIκBα

In vitro–translated FlagIκBα was immunoprecipitated by incubation with 10 μl of M2-agarose in 1 ml of Buffer B (10 mM Tris, pH 7.6, 100 mM NaCl, 0.1% NP-40, 10 μg/ml leupeptin, 1 mM DTT) with end-over-end rotation for 1 hr at 4°C. Resins were then washed three times with Buffer B, once with Buffer C (10 mM Tris, pH 7.6, 1 mg/ml BSA, 10 μg/ml leupeptin, 1 mM DTT), and then eluted by the addition of 24 μl of Buffer C containing 0.7 mg/ml Flag peptide for 30 min on Ice.

Western Blotting

Proteins were electrophoresed by SDS-PAGE and transferred to Immobilon-NC membranes (Millipore). The membranes were blocked with 5% nonfat milk and probed with rabbit anti-IκBα polyclonal antibodies (C21, Santa Cruz Biotechnology). Membranes were then incubated with goat anti-rabbit IgG-alkaline phosphatase or donkey anti-rabbit IgG-horseradish peroxidase conjugates, and developed using standard chromogenic or Enhanced Chemiluminescence (Amersham) substrates, respectively. Western blots of purified IκBα kinase employed antibodies (anti-MEKK1 [C22], anti-MKK4 [C20], anti-JNK1 [FL], anti-JNK2 [FL]) obtained from Santa Cruz Biotechnology.

Extract Preparation

HeLa S_3 cell cytoplasmic extracts were prepared by two methods. In the first ("rapid lysis procedure"), mid-logarithmic growth phase HeLa S_3 cells cultured in RPMI 1640 media supplemented with 5% horse serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin were centrifuged at 2,600 × g for 10 min. Cells were resuspended in RPMI media containing 5% horse serum, and then either mock treated or incubated with 1000 U/ml TNF-α at 37°C. At various times, cells were centrifuged at 1,000 × g for 1 min at ambient temperature. Cells were then rapidly washed with ice-cold PBS, centrifuged again at 1,000 × g for 1 min, resuspended in ice-cold 50 mM Tris (pH 7.5), 1 mM EGTA, and then immediately lysed by dounce homogenization (15–20 strokes with an A-type pestle). The crude lysate was clarified by centrifugation at 4,600 × g for 10 min at 4°C, and the resulting supernatant immediately frozen at −80°C. In the second method ("S100"), HeLa S_3 cells were swollen in a hypotonic buffer and lysed, followed by removal of nuclei and centrifugation at 100,000 × g as described (Dignam et al., 1983). The supernatant was then dialyzed extensively against 20 mM Tris

(pH 7.5), 0.5 mM DTT. If not employed immediately, the extract was stored at −80°C.

Purification of IκBα Kinase

HeLa cell S100 cytoplasmic extract, prepared as above, was applied to a Mono-Q anion exchange column. The IκBα kinase activity was eluted with 0.2–0.3 M KCl in Buffer D (50 mM Tris, pH 7.5, 0.5 mM DTT), and then precipitated with 40% ammonium sulfate. The resuspended precipitates were dialyzed against 10 mM K_2HPO_4-KH_2PO_4 (pH 7.0), 0.5 mM DTT, and then applied to a hydroxylapatite column. After elution with 0.2 M K_2HPO_4-KH_2PO_4 (pH 7.0), the kinase-containing fractions were applied to a Superdex-200 gel filtration column equilibrated with 50 mM Tris (pH 7.5), 0.5 mM DTT, and 150 mM NaCl. The high molecular weight fractions that contained the kinase activity were applied to a Mono-Q column and eluted with a linear gradient of 150–325 mM NaCl in Buffer D. Fractions from the Superdex-200 and second Mono-Q chromatographies were assayed for IκBα kinase activity in the presence of ubiquitination components (Ubc4 and ubiquitin, in addition to E1 supplied by the wheat germ extract employed for in vitro translation of IκBα) (Chen et al., 1996), or recombinant MEKK1Δ.

Preparation of Recombinant Proteins

(His)$_6$MEKK1Δ and (His)$_6$MEKK1Δ (K432M) were purified using Ni-NTA agarose from Sf9 cells infected with baculovirus prepared using the Bac-to-Bac Expression System (GIBCO-BRL Life Technologies). pFastBacHT-MEKK1Δ and pFastBacHT-MEKK1Δ (K432M) were constructed by subcloning the 1.2 kb NcoI/XbaI coding sequence fragment of pcDNA3-FlagMEKK1Δ and pcDNA3-FlagMEKK1Δ (K432M), respectively, into the NcoI/XbaI site of pFastBacHTa. Recombinant bacmids and baculovirus were subsequently prepared according to the manufacturer's instructions. GST-MKK4 and GST-JNK1 were purified from E. coli HB101 transformed with pGEX-MKK4 and pGEX-JNK1, respectively, employing glutathione agarose affinity chromatography as described (Smith and Johnson, 1988). pGEX-MKK4 was constructed by subcloning the 1.1 kb BamHI (blunt)/BspI20I (blunt) fragment of pcDNA3-FlagMKK4 containing the MKK4 coding sequence into the SmaI site of pGEX-3X (Pharmacia). pGEX-JNK1 was constructed by subcloning the 1.4 kb NcoI (blunt)/SalI fragment of pSRαHA-JNK1 containing the JNK1 coding sequence into the EcoRI (blunt)/SalI site of pGEX-5X-1 (Pharmacia). (His)$_6$-IκBα was purified using Ni-NTA agarose from E. coli BL21(DE3)LysS transformed with pRSET-IκBα. pRSET-IκBα was constructed by subcloning the EagI (blunt)/HindIII fragment of pBS-IκBα containing the IκBα coding sequence into the PvuII/HindIII site of pRSET A (Invitrogen). The E2 enzymes Ubc4 and GST-Ubc5 were prepared as described (Chen et al., 1996). Concentrations of recombinant proteins were determined by SDS-PAGE followed by staining with Coomassie blue and comparison with bovine serum albumin standards.

Protein Kinase Assays

Mobility Shift Assays

Typically, HeLa cell cytoplasmic extracts or purified IκBα kinase (from gel filtration chromatography as described above) was incubated with 0.5 μl of in vitro-translated, ^{35}S-labeled protein in a total volume of 10 μl containing 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP, 10 mM phosphocreatine, 3.5 U/ml creatine phosphokinase, and 2.5 μM okadaic acid. In vitro-translated, ^{35}S-labeled IκBα, Flag-IκBα, and c-Jun, or their phosphorylation defective mutants, were prepared using TnT wheat germ extract kits (Promega) and pBS-IκBα, pBS-IκBα (S32A/S36A), pBS-FlagIκBα, pBS-FlagIκBα (S32A/S36A), pcDNA1-c-Jun, or pcDNA1-cJun(S63A/S73A) as templates.

[γ-^{32}P]ATP Labeling of IκBα

Enzyme was incubated with 0.5 μg (His)$_6$IκBα in 10 μl of 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2.5 μM okadaic acid, 200 μM ATP and 5 μCi of [γ-^{32}P]ATP. Incubations were carried out at 30°C for 30 min.

Dephosphorylation of IκBα Kinase Complex

Purified IκBα kinase (from gel filtration chromatography) was treated with MEKK1Δ in 50 mM Tris (pH 7.6), 5 mM $MgCl_2$, 2 mM ATP for 30 min at 30°C. MEKK1Δ-activated IκBα kinase was separated from ATP by centrifugal gel filtration on Sephadex G50 and subsequently incubated with or without calf intestinal alkaline phosphatase (CIP) in 50 mM Tris (pH 7.8), 0.1 mM EDTA for 30 min at 30°C. IκBα kinase was then separated from CIP and MEKK1Δ by chromatography on a Superdex 200 column and assayed for IκBα kinase activity in the absence or presence of MEKK1Δ.

[γ-^{32}P]ATP Labeling of IκBα Kinase Complex

Two nanograms of MEKK1Δ was incubated in 7 μl of 70 mM Tris (pH 7.6), 7 mM $MgCl_2$, 3.5 μM okadaic acid, and 140 μM ATP for 15 min at 30°C. Subsequently, purified IκBα kinase (from the second Mono Q chromatography step as described above) and 10 μCi of [γ-^{32}P]ATP in a total volume of 3 μl were added and the incubation continued at 30°C for an additional 30 min. In control reactions, either MEKK1Δ or IκBα kinase was omitted.

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Commentary

JNK or IKK, AP-1 or NF-κB, which are the targets for MEK kinase 1 action?

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MEKK1 (MEK kinase 1) is a mammalian serine/threonine kinase in the mitogen-activated protein kinase (MAPK) kinase kinase (MAPKKK) group (1). Being the first mammalian homolog of STE11, a MAPKKK that activates the pheromone responsive MAPK cascade of budding yeast, MEKK1 as its name indicates was thought to be an activator of the MAPK kinase (MAPKK) MEK1/2 and thus an activator of the ERK MAPK cascade. It therefore was rather surprising that titration experiments (2) or analysis of cells engineered to express MEKK1 from an inducible promoter (3) revealed that it is a far more potent activator of the JNK MAPK cascade. These observations made by using either the catalytic domain of MEKK1 (MEKK1Δ) or a 672-aa C-terminal fragment recently were confirmed by using full-length human MEKK1 (Y. Xia, Z. Wu, B. Su, B. Murray, and M.K., unpublished work). Most importantly, when different mammalian MAPKKs were examined *in vitro* or *in vivo* for phosphorylation and activation by MEKK1, a MAPKK called JNKK1 (MKK4 or SEK1), whose function is JNK (and p38 MAPK) activation (4) was found to be the preferred MEKK1 substrate (Y. Xia, Z. Wu, B. Su, B. Murray, and M.K., unpublished work). Based on specificity constants, MEKK1 phosphorylates JNKK1 45-fold more efficiently than it phosphorylates MEK1/2 (Y. Xia, Z. Wu, B. Su, B. Murray, and M.K., unpublished work), thus providing a clear biochemical explanation for the marked pro-JNK bias of MEKK1. Targets for JNK include transcription factors c-Jun and ATF2, which are components of the AP-1 dimer that are involved in induction of the *c-jun* protooncogene (5). JNK-mediated phosphorylation enhances the transcriptional activity of both c-Jun and ATF2 (6, 7). Correspondingly, MEKK1 expression plasmids are potent activators of a chimeric c-Jun-GAL4 transcription factor, in which the c-Jun activation domain is fused to the GAL4 DNA binding domain (8). Overexpression of a catalytically inactive MEKK1(KM) mutant inhibits JNK activation by either epidermal growth factor (EGF) or tumor necrosis factor (TNF) (refs. 8 and 9 and Y. Xia, Z. Wu, B. Su, B. Murray, and M.K., unpublished work). This mutant was used to show that signals generated by occupancy of TNF type 1 receptor (TNF-R1) diverge downstream to the signaling proteins TRAF2 and RIP, which are recruited to TNF-R1, such that one pathway leads to JNK (and p38 MAPK) activation followed by stimulation of AP-1 activity and the other mediates NF-κB activation (10, 11) (Fig. 1). These experiments also demonstrated that NF-κB activation protects cells against TNF-induced apoptosis, whereas JNK (and p38) activation does not affect programmed cell death either positively or negatively. Similar results were obtained by analysis of mice and cells deficient in the RelA(p65) subunit of NF-κB (12, 13).

In light of these findings, it was somewhat surprising that under different circumstances overexpression of MEKK1 was found to stimulate NF-κB activity (14, 15). NF-κB is a dimeric transcription factor composed of Rel proteins whose activity is regulated through interaction with specific inhibitors, the IκBs (16–18). In response to cell stimulation the IκBs are rapidly phosphorylated and then undergo ubiquitin-mediated proteolysis, resulting in the release of active NF-κB dimers that translocate to the nucleus. Initially, the demonstration that MEKK1 overexpression leads to NF-κB activation was based solely on the use of an NF-κB transcriptional reporter. As there are ample examples for transcriptional synergy between AP-1 and NF-κB (19, 20), such results should be interpreted with caution. It is expected that a signaling pathway that enhances only AP-1 activity still may stimulate an NF-κB-dependent promoter, even in the absence of overt AP-1 binding sites. Likewise, an AP-1-dependent promoter may respond to NF-κB even in the absence of recognizable NF-κB binding sites. In light of these limitations, a bigger surprise were the results of Lee *et al.* (21) who reported that addition of recombinant MEKK1Δ to a partially enriched fraction of non-stimulated HeLa cells stimulated a protein kinase activity that phosphorylated IκBα at serines (S) 32 and 36, sites that previously were shown to be phosphorylated in response to cell stimulation with TNF or interleukin 1 (IL-1). Phosphorylation at S32 and S36 results in polyubiquitination and degradation of IκBα (22, 23). Homologous phosphoacceptor sites are essential for the induced degradation of other IκB proteins (23). As activation of an IκB kinase by MEKK1Δ was demonstrated by using a rather crude fraction whose polypeptide composition was not described, the identity of this activity remained a mystery. In the meantime, two other groups working independently have succeeded in purifying an inducible IκB kinase activity from extracts of TNF-stimulated HeLa or Jurkat cells (24, 25). Extensive purification of that activity, named IKK, which elutes from gel filtration columns as a large complex with an apparent molecular mass of 700–900 kDa, revealed the presence of two polypeptides with molecular masses of 85 and 87 kDa that precisely coeluted with IκB kinase activity. Microsequencing and molecular cloning revealed that these polypeptides are closely related protein kinases named IKKα (or IKK1) and IKKβ (or IKK2), respectively (24–26). IKKα and IKKβ also were identified through a different approach, based on yeast two-hybrid screens, as proteins that interact with a MAPKKK called NIK (NF-κB-inducing kinase) (27, 28). NIK originally was identified as a TRAF2-interacting kinase whose overexpression results in potent NF-κB activation (29) without any considerable effect on MAPKs, including JNK (30). Therefore the observed interaction between NIK and the IKKs immediately suggested that NIK may be an upstream activator of IKK (Fig. 1). Although the IKK complex is similar in size to the MEKK1Δ-responsive activity, the relationships between the two remained nebulous, and various attempts to stimulate IKK activity with modest amounts of MEKK1Δ expression vector, that are sufficient for JNK activation, have failed (24) (D. Goeddel, personal communication). In addition, several reports indicating that NF-κB transcriptional reporters are not stimulated by low to modest doses of MEKK1 (which are sufficient for JNK activation), while being highly responsive to co-

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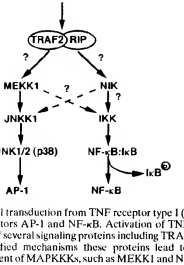

FIG. 1. Signal transduction from TNF receptor type I (TNF-R1) to transcription factors AP-1 and NF-κB. Activation of TNF-R1 results in recruitment of several signaling proteins including TRAF2 and RIP. By yet unidentified mechanisms these proteins lead to activation and/or recruitment of MAPKKKs, such as MEKK1 and NIK. MEKK1 is responsible for direct activation of JNKK1, a MAPKK that directly activates JNK1/2 and p38 MAPK, thereby leading to stimulation of transcription factor AP-1. MEKK1 also may be involved in NF-κB activation. NIK or a closely related family member leads to activation of the IKK complex, which leads to phosphorylation of IκBs, thereby triggering their degradation. This results in activation of NF-κB.

transfected NIK, have appeared (30, 31). However, in new work published in this issue of the *Proceedings*, Lee *et al.* (32) present evidence that the MEKK1Δ-responsive activity they previously identified is none other than the cytokine-responsive IKK. Furthermore, they suggest that MEKK1 may be a direct activator of IKKα and IKKβ.

That IKK activity is regulated through phosphorylation of some of its subunits previously was demonstrated by the use of protein phosphatase 2A (PP2A) catalytic subunit, whose incubation with purified IKK resulted in loss of IκB kinase activity (24). Furthermore, coexpression with NIK stimulates the kinase activity of transiently expressed IKKα, which is also efficiently phosphorylated *in vitro* by NIK immunoprecipitates (33). Although no specificity constants were determined, IKKβ appears to be a relatively poor NIK substrate (33). Analysis of the IKKα and IKKβ protein sequences reveals several potential phosphoacceptor sites in a region conserved in all protein kinases, the T (or activation) loop, that resemble those that are used by MAPKKKs to activate MAPKKs (25). Indirect evidence that these sites may be used to activate IKKα and IKKβ was provided by site-directed mutagenesis (25, 33), but so far these sites were not shown to be phosphorylated in TNF or IL-1 stimulated cells or be involved in cytokine-mediated IKK activation. Substitution of S176 in IKKα with alanine was found to decrease its phosphorylation and activation by NIK (33), whereas a dual substitution of S177 and S181 of IKKβ with glutamic acid was reported to increase its catalytic activity (25). The current work (32) shows that recombinant MEKK1Δ can phosphorylate a synthetic peptide corresponding to the T loop of IKKβ and that substitution of S177 and S181 with alanines reduces the extent of ^{32}P incorporation (32). It also is shown that incubation of a partially purified preparation with MEKK1Δ results in phosphorylation of two polypeptides whose sizes match those of IKKα and IKKβ (32). However, as these and similar experiments conducted with NIK have not been performed with fully purified proteins the results fall short of a conclusive demonstration that MEKK1 or NIK can directly phosphorylate and activate native IKKα and IKKβ. Nevertheless, the simplest interpretation of past and present results is that either of these MAPKKKs can activate IKK.

An important question, however, that is yet to be answered is which MAPKKKs are physiologically involved in IKK and NF-κB activation and whether different NF-κB-activating stimuli use the same MAPKKKs. It is also to be resolved whether MEKK1 acts exclusively on the JNK (and p38) to AP-1 pathway or whether it also is involved in IKK and NF-κB activation. In this respect, it would be useful to compare whether the specificity constants for IKKα or IKKβ phosphorylation by MEKK1 match the one for JNKK1, the most efficient and relevant MEKK1 substrate identified so far. Most groups who cotransfected varying amounts of truncated MEKK1 expression vectors with either a JNK reporter plasmid or an NF-κB transcriptional reporter find that JNK activity is potently stimulated at low input levels whereas NF-κB transcriptional activity is stimulated only by very high doses of MEKK1 (10, 30–32). High doses of MEKK1 are known to have nonspecific effects (2). Activation of a GAL4 transcriptional reporter by the c-Jun-GAL4 chimera and cotransfected MEKK1 parallels the stimulation of JNK activity (8, 34), but a different AP-1 reporter containing multiple c-Jun:ATF2 binding sites is stimulated only by very high doses of MEKK1, similar to those required for stimulation of the NF-κB reporter (32). Although all groups seem to agree that cotransfection of a NIK expression vector has no effect on JNK activity, some find that NIK is nevertheless can enhance AP-1 activity, albeit less efficiently than MEKK1 (31). Currently it is hard to reconcile all of these results even if one invokes transcriptional synergy between AP-1 and NF-κB. More puzzling differences are found when the abilities of MEKK1Δ and NIK to activate NF-κB and IKK are compared. All groups agree that MEKK1 is a much poorer activator of the NF-κB transcriptional reporter than NIK is (30–32). However, some find that MEKK1 and NIK expression plasmids have similar effects on the activity of transiently expressed IKKα (32), and others find that NIK is a much more potent activator of IKKα than MEKK is, whereas IKKβ is slightly more responsive to MEKK1 than to NIK (35). A major difference between measuring the response of an NF-κB transcriptional reporter to MEKK1 vs. activation of a transiently expressed epitope tagged IKKα or IKKβ is that in the former case NF-κB activation depends on stimulation of endogenous (physiological) IKK activity, whereas in the latter case the transiently overexpressed IKK subunit probably is not incorporated into the physiological IKK complex. In fact, protein purification and immunoprecipitation experiments strongly suggest that most of the IKK complexes are IKKα:IKKβ heterodimers plus additional subunits and that very little IKKα or IKKβ homodimeric complexes exist (E. Zandi, D. Rothwarf, and M.K., unpublished results). It is important to express only small amounts of exogenous IKKα or IKKβ to ensure their incorporation into the physiological 900-kDa IKK complex (26). It is therefore safer to compare the abilities of NIK and MEKK1 to activate the endogenous IKK complex rather than the artificial IKKα or IKKβ homodimers generated by transient overexpression. When such a comparison is performed, transient transfection of a NIK vector into 293 cells results in preferential activation of endogenous IKK whereas transfection of a full-length MEKK1 vector results in preferential JNK activation (Fig. 2*A*). However overexpression of NIK can lead to JNK activation whereas overexpression of MEKK1 can lead to IKK activation (Fig. 2*B*). These results are in complete agreement with all published comparisons of the effect of these MAPKKKs on NF-κB and AP-1 transcriptional reporters. Nevertheless, it should be realized that these results do not rule out the possibility that, although weak, MEKK1 may contribute to IKK activation nor do they prove that, although potent, NIK is a physiological NF-κB activator. In addition these experiments highlight the potential pitfalls associated with overexpression of signaling proteins.

In addition to proinflammatory cytokines IKK activity is potently stimulated by the Tax transactivator protein of human T cell leukemia virus (HTLV) (36, 37). This response leading to NF-κB activation is likely to play a major role in the leukemogenic

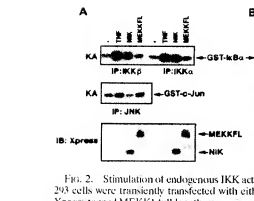

FIG. 2. Stimulation of endogenous IKK activity by NIK and MEKK1. 293 cells were transiently transfected with either Xpress-tagged NIK or Xpress-tagged MEKK1 full-length expression vectors (1 μg DNA in A or 2 μg DNA in B/60-mm plate). After 24 hr, cells were treated or not with TNF (20 ng/ml for 10 min) and then lysed. Cell lysates were immunoprecipitated (IP) with either anti-IKKα or anti-IKKβ or anti-JNK antibodies. The IKK activity (KA) was determined by using glutathione *S*-transferase (GST)-IκBα (1–54) as a substrate. The JNK activity (KA) was determined by using GST-cJun (1–79) as a substrate.

function of this virus. Like proinflammatory cytokines Tax was proposed to act either via MEKK1 (37) or NIK (36). Although the strongest evidence in favor of MEKK1 as a target is based on its ability to physically interact with Tax (37), the pro-NIK evidence is based on genetic arguments (36). Uhlik *et al.* (36) isolated variants of the Jurkat T cell line that fail to activate NF-κB in response to Tax. Although the basis for this defect currently is unknown, it can be complemented by transient expression of NIK but not by MEKK1 overexpression (36).

A major problem in sorting out the exact physiological functions of these and other MAPKKKs is the difficulty in detecting considerable changes in their enzymatic activity in response to cell stimulation by using conventional immunoprecipitation experiments. This deficiency can be overcome by genetic experiments similar to those that established the function of the yeast MAPKKK STE11 in three distinct MAPK cascades (38, 39). Although we will have to await the results of gene knockout experiments in which the activities of NIK and MEKK1 are selectively abolished, similar experiments conducted with components of the TNF-RI response pathway clearly support the earlier conclusion (10) that the pathways leading from this receptor to either JNK and AP-1 or IKK and NF-κB diverge at the level of TRAF2 and RIP (Fig. 1). Cells established from TRAF2 knockout embryos are defective in JNK activation in response to TNF, while exhibiting only a slightly retarded NF-κB activation response (40). In contrast, cells derived from RIP knockout embryos are defective in NF-κB activation, while exhibiting a normal JNK activation response (41). As MAPKKKs, like MEKK1 and NIK, are thought to act downstream to TRAF2 and RIP rather than upstream to them, it is unlikely that they play equal roles in transducing signals generated by TNF-RI activation to transcription factors. It is also possible that neither MEKK1 nor NIK are involved in TNF signaling. After all, the MAPKKK family contains many other members in addition to these two usual suspects.

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